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(54) Title: HYBRID ADENOVIRUS-AAV VIRUS AND METHOD OF USE THEREOF

(57) Abstract

The present invention provides a hybrid vector construct which comprises a portion of an adenovirus, 5' and 3' ITR sequences from an AAV, and a selected transgene. Also provided is a hybrid virus linked via a polycation conjugate to an AAV *rep* gene to form a single particle. These trans-infection particles are characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome. Also disclosed is the use of the hybrid vectors and viruses to produce large quantities of recombinant AAV.

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HYBRID ADENOVIRUS-AAV VIRUS AND METHODS OF USE THEREOF

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Field of the Invention

The present invention relates to the field of vectors useful in somatic gene therapy and the production thereof.

Background of the Invention

Recombinant adenoviruses are capable of providing extremely high levels of transgene delivery to virtually all cell types, regardless of the mitotic state. High titers (10^{13} plaque forming units/ml) of recombinant virus can be easily generated in 293 cells (the adenovirus equivalent to retrovirus packaging cell lines) and cryo-stored for extended periods without appreciable losses.

The primary limitation of this virus as a vector resides in the complexity of the adenovirus genome. A human adenovirus is comprised of a linear, approximately 36 kb double-stranded DNA genome, which is divided into 100 map units (m.u.), each of which is 360 bp in length. The DNA contains short inverted terminal repeats (ITR) at each end of the genome that are required for viral DNA replication. The gene products are organized into early (E1 through E4) and late (L1 through L5) regions, based on expression before or after the initiation of viral DNA synthesis [see, e.g., Horwitz, Virology, 2d edit., ed. B. N. Fields, Raven Press, Ltd. , New York (1990)].

A human adenovirus undergoes a highly regulated program during its normal viral life cycle [Y. Yang et al, Proc. Natl. Acad. Sci., USA, 91:4407-4411 (1994)].

virions are internalized by receptor-mediated endocytosis and transported to the nucleus where the immediate early genes, E1a and E1b, are expressed. Because these early gene products regulate expression of a variety of host genes (which prime the cell for virus production) and are central to the cascade activation of early delayed genes (e.g. E2, E3, and E4) followed by late genes (e.g. L1-5), first generation recombinant adenoviruses for gene therapy focused on the removal of the E1 domain. This strategy was successful in rendering the vectors replication defective, however, *in vivo* studies revealed transgene expression was transient and invariably associated with the development of severe inflammation at the site of vector targeting [S. Ishibashi et al, J. Clin. Invest., 93:1885-1893 (1994); J. M. Wilson et al, Proc. Natl. Acad. Sci., USA, 85:4421-4424 (1988); J. M. Wilson et al, Clin. Bio., 3:21-26 (1991); M. Grossman et al, Som. Cell. and Mol. Gen., 17:601-607 (1991)].

Adeno-associated viruses (AAV) have also been employed as vectors. AAV is a small, single-stranded (ss) DNA virus with a simple genomic organization (4.7 kb) that makes it an ideal substrate for genetic engineering. Two open reading frames encode a series of *rep* and *cap* polypeptides. *Rep* polypeptides (*rep*78, *rep*68, *rep*62 and *rep*40) are involved in replication, rescue and integration of the AAV genome. The *cap* proteins (VP1, VP2 and VP3) form the virion capsid. Flanking the *rep* and *cap* open reading frames at the 5' and 3' ends are 145 bp inverted terminal repeats (ITRs), the first 125 bp of which are capable of forming Y- or T-shaped duplex structures. Of importance for the development of AAV vectors, the entire *rep* and *cap* domains can be excised and replaced with a therapeutic or reporter transgene [B. J. Carter, in "Handbook of Parvovirus", ed., P. Tijsser, CRC Press, pp.155-168

(1990)]. It has been shown that the ITRs represent the minimal sequence required for replication, rescue, packaging, and integration of the AAV genome.

The AAV life cycle is biphasic, composed of both latent and lytic episodes. During a latent infection, AAV virions enter a cell as an encapsidated ssDNA, and shortly thereafter are delivered to the nucleus where the AAV DNA stably integrates into a host chromosome without the apparent need for host cell division. In the absence of helper virus, the integrated ss DNA AAV genome remains latent but capable of being activated and rescued. The lytic phase of the life cycle begins when a cell harboring an AAV provirus is challenged with a secondary infection by a herpesvirus or adenovirus which encodes helper functions that are recruited by AAV to aid in its excision from host chromatin [B. J. Carter, cited above]. The infecting parental ssDNA is expanded to duplex replicating form (RF) DNAs in a *rep* dependent manner. The rescued AAV genomes are packaged into preformed protein capsids (icosahedral symmetry approximately 20 nm in diameter) and released as infectious virions that have packaged either + or - ss DNA genomes following cell lysis.

Progress towards establishing AAV as a transducing vector for gene therapy has been slow for a variety of reasons. While the ability of AAV to integrate in quiescent cells is important in terms of long term expression of a potential transducing gene, the tendency of the integrated provirus to preferentially target only specific sites in chromosome 19 reduces its usefulness. Additionally, difficulties surround large-scale production of replication defectiv recombinants. In contrast to the production of recombinant retrovirus or adenovirus, the only widely recognized means for manufacturing transducing AAV virions entails co-

transfection with two different, yet complementing plasmids. One of these contains the therapeutic or reporter minigene sandwiched between the two *cis* acting AAV ITRs. The AAV components that are needed for rescue and subsequent packaging of progeny recombinant genomes are provided in *trans* by a second plasmid encoding the viral open reading frames for *rep* and *cap* proteins. The cells targeted for transfection must also be infected with adenovirus thus providing the necessary helper functions. Because the yield of recombinant AAV is dependent on the number of cells that are transfected with the *cis* and *trans*-acting plasmids, it is desirable to use a transfection protocol with high efficiency. For large-scale production of high titer virus, however, previously employed high efficiency calcium phosphate and liposome systems are cumbersome and subject to inconsistencies.

There remains a need in the art for the development of vectors which overcome the disadvantages of the known vector systems.

Summary of the Invention

In one aspect, the present invention provides a unique recombinant hybrid adenovirus/AAV virus, which comprises an adenovirus capsid containing selected portions of an adenovirus sequence, 5' and 3' AAV ITR sequences which flank a selected transgene under the control of a selected promoter and other conventional vector regulatory components. This hybrid virus is characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome in the presence of the *rep* gene. In one embodiment, th transgene is a reporter gene. Another embodim nt of the hybrid virus contains a therapeutic transgene. In a pr ferred embodiment, the

hybrid virus has associated therewith a polycation sequence and the AAV *rep* gene. This construct is termed the hybrid virus conjugate or trans-infection particle.

In another aspect, the present invention provides a hybrid vector construct for use in producing the hybrid virus or viral particle described above. This hybrid vector comprises selected portions of an adenovirus sequence, 5' and 3' AAV ITR sequences which flank a selected transgene under the control of a selected promoter and other conventional vector regulatory components.

In another aspect, the invention provides a composition comprising a hybrid viral particle for use in delivering a selected gene to a host cell. Such a composition may be employed to deliver a therapeutic gene to a targeted host cell to treat or correct a genetically associated disorder or disease.

In yet another aspect, the present invention provides a method for producing the hybrid virus by transfecting a suitable packaging cell line with the hybrid vector construct of this invention. In another embodiment the method involves co-transfecting a cell line (either a packaging cell line or a non-packaging cell line) with a hybrid vector construct and a suitable helper virus.

In a further aspect, the present invention provides a method for producing large quantities of recombinant AAV particles with high efficiency by employing the above methods, employing the hybrid vector construct of this invention and collecting the rAAV particles from a packaging cell line transfected with the vector.

Other aspects and advantages of the pres nt invention are d scribed further in the following detailed d scription of the preferred mbodiments thereof.

Brief Description of the Drawings

Fig. 1A is a schematic diagram of a vector construct pAd.AV.CMV*LacZ* [SEQ ID NO: 1], which contains (from the top in clockwise order) adenovirus sequence map units 0-1 (clear bar); the 5' AAV ITR (solid bar); a CMV immediate early enhancer/promoter (hatched arrow), an SV40 intron (clear bar), an *E. coli* beta-galactosidase cDNA (*LacZ*) (hatched line), an SV40 polyadenylation signal (clear bar), a 3' AAV ITR (solid bar), adenovirus sequence from map units 9-16 (clear bar), and a portion of a pBR322 derivative plasmid (thin solid line). Restriction endonuclease enzymes are identified by their conventional designations; and the location of each restriction enzyme is identification by the nucleotide number in parentheses to the right of the enzyme designation.

Fig. 1B is a schematic drawing demonstrating linearization of pAd.AV.CMV*LacZ* [SEQ ID NO: 1] by digestion with restriction enzyme NheI and a linear arrangement of a ClaI digested adenovirus type 5 with deletions from mu 0-1. The area where homologous recombination will occur (between m.u. 9-16) in both the plasmid and adenovirus sequences is indicated by crossed lines.

Fig. 1C is a schematic drawing which demonstrates the hybrid virus Ad.AV.CMV*LacZ* after co-transfection of the linearized pAd.AV.CMV*LacZ* [SEQ ID NO: 1] and adenovirus into 293 cells followed by intracellular homologous recombination.

Fig. 2A-2K report the top DNA strand of the double-strand plasmid pAd.AV.CMV*LacZ* [SEQ ID NO: 1] (the complementary strand can be readily derived by one of skill in the art). With reference to SEQ ID NO: 1, nucleotides 1-365 are adenovirus type 5 sequences; the 5' AAV ITR sequence spans nucleotides 366-538; the CMV

promoter/enhancer spans nucleotides 563-1157; the SV-40 intron spans nucleotides 1158-1179; the *LacZ* gene spans nucleotides 1356-4827; the SV-40 poly A sequence spans nucleotides 4839-5037; the 3' AAV ITR spans nucleotides 5053 to 5221; nucleotides 5221 to about 8100 are adenovirus type 5 sequences. The remaining sequences are non-specific/plasmid sequences.

Fig. 3 is a bar graph plotting u.v. absorbance at 420 nm of the beta-galactosidase blue color for a control and ten putative positive clones (D1A through D1J) of 293 cells transfected with the recombinant hybrid Ad.AV.CMV*LacZ*. Eight of the clones expressed high levels of enzyme.

Fig. 4 is a schematic diagram of pRep78/52 [SEQ ID NO: 2]. This plasmid includes an AAV P5 promoter, Rep78, Rep52 and a poly-A sequence in a pUC18 plasmid background.

Figs. 5A - 5E report nucleotides 1-4910 of the top DNA strand of the double-strand plasmid pRep78/52 [SEQ ID NO: 2] (the complementary strand can be readily derived by one of skill in the art).

Fig. 6 is a flow diagram of the construction of a trans-infection particle formed by a hybrid virus, a poly-L-lysine sequence and attached AAV *rep*-containing plasmid.

Fig. 7 is a flow diagram of the hybrid virus' life cycle, in which a trans-infection particle enters the cell and is transported to the nucleus. The virus is uncoated and the *rep* mediates rescue of the inserted gene, which is then integrated into the chromosome of the host cell.

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The present invention provides a hybrid vector construct which comprises a portion of an adenovirus, 5' and 3' ITR sequences from an AAV, and a selected transgene. Also provided is a hybrid virus linked via a polycation conjugate to an AAV *rep* gene to form a single particle. These trans-infection particles are characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome. Also disclosed is the use of the hybrid vectors and viruses to produce large quantities of recombinant AAV.

I. Construction of the Hybrid Vector and Virus

A. *The Adenovirus Component of the Vector and Virus*

The hybrid virus of this invention uses adenovirus nucleic acid sequences as a shuttle to deliver a recombinant AAV/transgene genome to a target cell. The DNA sequences of a number of adenovirus types, including type Ad5, are available from Genbank. The adenovirus sequences may be obtained from any known adenovirus type, including the presently identified 41 human types [Horwitz et al, cited above]. Similarly adenoviruses known to infect other animals may also be employed in the vector constructs of this invention. The selection of the adenovirus type is not anticipated to limit the following invention. A variety of adenovirus strains are available from the American Type Culture Collection, Rockville, Maryland, or available by request from a variety of commercial and institutional sources. In the following exemplary embodiment an adenovirus, type 5 (Ad5) is used for convenience.

The adenovirus nucleic acid sequences employed in the hybrid vector of this invention can range from a minimum sequence amount, which requires the use of a helper virus to produce the hybrid virus particle, to only selected deletions of adenovirus genes, which deleted gene products can be supplied in the hybrid viral production process by a selected packaging cell. Specifically, at a minimum, the adenovirus nucleic acid sequences employed in the pAdΔ shuttle vector of this invention are adenovirus genomic sequences from which all viral genes are deleted and which contain only those adenovirus sequences required for packaging adenoviral genomic DNA into a preformed capsid head. More specifically, the adenovirus sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR)

sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. According to this invention, the entire adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region can be employed as the 5' adenovirus sequence in the hybrid virus. This left terminal (5') sequence of the Ad5 genome useful in this invention spans bp 1 to about 360 of the conventional adenovirus genome, also referred to as map units 0-1 of the viral genome, and generally is from about 353 to about 360 nucleotides in length. This sequence includes the 5' ITR (bp 1-103 of the adenovirus genome); and the packaging/enhancer domain (bp 194-358 of the adenovirus genome). Preferably, this native adenovirus 5' region is employed in the hybrid virus and vector in unmodified form. Alternatively, corresponding sequences from other adenovirus types may be substituted. These Ad sequences may be modified to contain desired deletions, substitutions, or mutations, provided that the desired function is not eliminated.

The 3' adenovirus sequences of the hybrid virus include the right terminal (3') ITR sequence of the adenoviral genome spanning about bp 35,353 - end of the adenovirus genome, or map units ~98.4-100. This sequence is generally about 580 nucleotide in length. This entire sequence is desirably employed as the 3' sequence of a hybrid virus. Preferably, the native adenovirus 3' region is employed in the hybrid virus in unmodified form. However, as described above with respect to the 5' sequences, some modifications to these sequences which do not adversely eff ct their biological function may be acceptable. As described below, wh n these 5' and 3' adenovirus sequences ar employed in the hybrid vector, a helper adenovirus which supplies all other essential

genes for viral formation alone or with a packaging cell line is required in the production of the hybrid virus or viral particle.

Alternative embodiments of the hybrid virus employ adenovirus sequences in addition to the minimum sequences, but which contain deletions of all or portions of adenovirus genes. For example, the adenovirus immediate early gene E1a (which spans mu 1.3 to 4.5) and delayed early gene E1b (which spans mu 4.6 to 11.2) should be deleted from the adenovirus sequence which forms a part of the hybrid vector construct and virus. Alternatively, if these sequences are not completely eliminated, at least a sufficient portion of the E1a and E1b sequences must be deleted so as to render the virus replication defective. These deletions, whether complete or partial, which eliminate the biological function of the gene are termed "functional deletions" herein.

Additionally, all or a portion of the adenovirus delayed early gene E3 (which spans mu 76.6 to 86.2) may be eliminated from the adenovirus sequence which forms a part of the hybrid virus. The function of E3 is irrelevant to the function and production of the hybrid virus.

All or a portion of the adenovirus delayed early gene E2a (which spans mu 67.9 to 61.5) may be eliminated from the hybrid virus. It is also anticipated that portions of the other delayed early genes E2b (which spans mu 29 to 14.2) and E4 (which spans mu 96.8 to 91.3) may also be eliminated from the hybrid virus and from the vector.

Deletions may also be made in any of the late genes L1 through L5, which span mu 16.45 to 99 of the adenovirus g nome. Similarly, deletions may be useful in the intermediate genes IX which maps between mu

9.8 and 11.2 and IVa_2 which maps between 16.1 to 11.1. Other deletions may occur in the other structural or non-structural adenovirus.

The above discussed deletions may occur individually, i.e., an adenovirus sequence for use in the present invention may contain deletions of E1 only. Alternatively, deletions of entire genes or portions effective to destroy their biological activity may occur in any combination. For example, in one exemplary hybrid vector, the adenovirus sequence may contain deletions of the E1 genes and the E3 gene, or of the E1, E2a and E3 genes, or of the E1 and E4 genes, or of E1, E2a and E4 genes, with or without deletion of E3, and so on.

The more deletions in the adenovirus sequence up to the minimum sequences identified above that characterize the hybrid virus, the larger the sequence(s) of the other below-described components to be inserted in the hybrid vector. As described above for the minimum adenovirus sequences, those gene sequences not present in the adenovirus portion of the hybrid virus must be supplied by either a packaging cell line and/or a helper adenovirus to generate the hybrid virus.

In an exemplary hybrid virus of this invention which is described below and in Example 1, the adenovirus genomic sequences present are from mu 0 to 1, mu 9 to 78.3 and mu 86 to 100 (deleted sequences eliminate the E1a and E1b genes and a portion of the E3 gene). From the foregoing information, it is expected that one of skill in the art may construct hybrid vectors and viruses containing more or less of the adenovirus gene sequence.

The portions of the adenovirus genome in the hybrid virus permit high production titers of the virus to be produced, often greater than $1x10^{13}$ pfu/ml. This is in stark contrast to the low titers ($1x10^{6}$ pfu/ml) that have been found for recombinant AAV.

B. *The AAV Components of the Vector and Virus*

Also part of the hybrid vectors and viruses of this invention are sequences of an adeno-associated virus. The AAV sequences useful in the hybrid vector are the viral sequences from which the *rep* and *cap* polypeptide encoding sequences are deleted. More specifically, the AAV sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. As stated above, the ITR sequences are about 143 bp in length. Substantially the entire sequences encoding the ITRs are used in the vectors, although some degree of minor modification of these sequences is expected to be permissible for this use. See, e.g., WO 93/24641, published December 9, 1993. The ability to modify these ITR sequences is within the skill of the art. For suitable techniques, see, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989).

The AAV ITR sequences may be obtained from any known AAV, including presently identified human AAV types. Similarly, AAVs known to infect other animals may also be employed in the vector constructs of this invention. The selection of the AAV is not anticipated to limit the following invention. A variety of AAV strains, types 1-4, are available from the American Type Culture Collection or available by request from a variety of commercial and institutional sources. In the following exemplary embodiment an AAV-2 is used for convenience.

In the hybrid vector construct, the AAV sequences are flanked by the selected adenovirus sequences discussed above. The 5' and 3' AAV ITR sequences themselves flank a selected transgene sequence

and associated regulatory elements, described below. Thus, the sequence formed by the transgene and flanking 5' and 3' AAV sequences may be inserted at any deletion site in the adenovirus sequences of the vector. For example, the AAV sequences are desirably inserted at the site of the deleted E1a/E1b genes of the adenovirus, i.e., after map unit 1. Alternatively, the AAV sequences may be inserted at an E3 deletion, E2a deletion, and so on. If only the adenovirus 5' ITR/packaging sequences and 3' ITR sequences are used in the hybrid virus, the AAV sequences are inserted between them.

C. The Transgene Component of the Hybrid Vector and Virus

The transgene sequence of the vector and recombinant virus is a nucleic acid sequence or reverse transcript thereof, heterologous to the adenovirus sequence, which encodes a polypeptide or protein of interest. The transgene is operatively linked to regulatory components in a manner which permits transgene transcription.

The composition of the transgene sequence will depend upon the use to which the resulting hybrid vector will be put. For example, one type of transgene sequence includes a reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include without limitation an *E. coli* beta-galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene and a green fluorescent protein gene. These sequences, when associated with regulatory elements which drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc.

Anoth r type of transgene sequence includes a therapeutic g ne which expresses a desired gene product in a host cell. These therapeutic genes or

nucleic acid sequences typically encode products for administration and expression in a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease. Such therapeutic genes which are desirable for the performance of gene therapy include, without limitation, a normal cystic fibrosis transmembrane regulator (CFTR) gene, a low density lipoprotein (LDL) gene, and a number of genes which may be readily selected by one of skill in the art. The selection of the transgene is not considered to be a limitation of this invention, as such selection is within the knowledge of those skilled in the art.

D. Regulatory Elements of the Hybrid Vector

In addition to the major elements identified above for the hybrid vector, i.e., the adenovirus sequences, AAV sequences and the transgene, the vector also includes conventional regulatory elements necessary to drive expression of the transgene in a cell transfected with the hybrid vector. Thus the vector contains a selected promoter which is linked to the transgene and located, with the transgene, between the AAV ITR sequences of the vector.

Selection of the promoter is a routine matter and is not a limitation of the hybrid vector itself. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable control of the amount of the transgene to be expressed. For example, a desirable promoter is that of the cytomegalovirus immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. Other desirable promot rs include, without limitation, the Rous sarcoma virus LTR promoter/enhancer and the chicken β-actin promoter. Still other promoter/enhancer sequences may be selected by one f skill in the art.

The vectors will also desirably contain nucleic acid sequences heterologous to the adenovirus sequences including sequences providing signals required for efficient polyadenylation of the transcript and introns with functional splice donor and acceptor sites. A common poly-A sequence which is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted in the vector following the transgene sequences and before the 3' AAV ITR sequence. A common intron sequence is also derived from SV-40, and is referred to as the SV-40 T intron sequence. A hybrid vector of the present invention may also contain such an intron, desirably located between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional and many such sequences are available [see, e.g., Sambrook et al, and references cited therein]. The DNA sequences encoding such regulatory regions are provided in the plasmid sequence of Fig. 2 [SEQ ID NO: 1].

The combination of the transgene, promoter/enhancer, the other regulatory vector elements and the flanking 5' and 3' AAV ITRs are referred to as a "minigene" for ease of reference herein. As above stated, the minigene is located in the site of any selected adenovirus deletion in the hybrid virus. The size of this minigene depends upon the amount and number of adenovirus sequence deletions referred to above. Such a minigene may be about 8 kb in size in the exemplary virus deleted in the E1 and E3 genes, as described in the examples below. Alternatively, if only the minimum adenovirus sequenc s are employed in the virus, this minigene may be a size up to about 30 kb. Thus, this hybrid vector and vector p rmit a gr at deal of latitude in the selection of the various components of the

minigene, particularly the transgene, with regard to size. Provided with the teachings of this invention, the design of such a minigene can be made by resort to conventional techniques.

E. Hybrid Vector Assembly and Production of Hybrid Virus

The material from which the sequences used in the hybrid vector, helper viruses, if needed, and recombinant hybrid virus (or viral particle) are derived and the various vector components and sequences employed in the construction of the hybrid vectors of this invention are obtained from commercial or academic sources based on previously published and described materials. These materials may also be obtained from an individual patient or generated and selected using standard recombinant molecular cloning techniques known and practiced by those skilled in the art. Any modification of existing nucleic acid sequences forming the vectors and viruses, including sequence deletions, insertions, and other mutations are also generated using standard techniques.

Assembly of the selected DNA sequences of the adenovirus, the AAV and the reporter genes or therapeutic genes and other vector elements into the hybrid vector and the use of the hybrid vector to produce a hybrid virus utilize conventional techniques, such as described in Example 1. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, cited above], use of overlapping oligonucleotide sequences of the adenovirus and AAV genomes, polymerase chain reaction, and any suitable method which provides the desired nucleotide sequence. Standard transf ction and co-transfection techniques are employ d, e.g., $CaPO_4$ transfection techniques using the complementation human embryonic

kidney (HEK) 293 cell line (a human kidney cell line containing a functional adenovirus E1a gene which provides a transacting E1a protein). Other conventional methods employed in this invention include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

As described in detail in Example 1 below and with resort to Fig. 1, a unique hybrid virus of this invention is prepared which contains an E1-deleted, partially E3 deleted, adenovirus sequence associated with a single copy of a recombinant AAV having deletions of its *rep* and *cap* genes and encoding a selected reporter transgene. Briefly, this exemplary hybrid virus was designed such that the AV.CMV*LacZ* sequence [SEQ ID NO: 1] (a minigene containing a 5'AAV ITR, a CMV promoter, an SV-40 intron, a *LacZ* transgene, an SV-40 poly-A sequence and a 3' AAV ITR) was positioned in place of the adenovirus type 5 (Ad5) E1a/E1b genes, making the adenovirus vector replication defective.

Because of the limited amount of adenovirus sequence present in the hybrid vectors of this invention, including the pAV.CMV*LacZ* [SEQ ID NO: 1] above, a packaging cell line or a helper adenovirus or both may be necessary to provide sufficient adenovirus gene sequences necessary for a productive viral infection to generate the hybrid virus.

Helper viruses useful in this invention contain selected adenovirus gene sequences not present in the hybrid vector construct or expressed by the cell line in which the hybrid vector is transfected. Optionally, such a helper virus may contain a second reporter minigene which enables s paration of th resulting hybrid virus and the helper virus upon purification. The

construction of desirable helper viruses is within the skill of the art.

As one example, if the cell line employed to produce the recombinant virus is not a packaging cell line, and the hybrid vector contains only the minimum adenovirus sequences identified above, the helper virus may be a wild type Ad virus. Thus, the helper virus supplies the necessary adenovirus early genes E1, E2a, E4 and all remaining late, intermediate, structural and non-structural genes of the adenovirus genome. However, if, in this situation, the packaging cell line is 293, which supplies the E1 proteins, the helper virus need not contain the E1 gene.

In another embodiment, when the hybrid construct is rendered replication defective by a functional deletion in E1 but contains no other deletions in Ad genes necessary for production of an infective viral particle, and the 293 cell line is employed, no helper virus is necessary for production of the hybrid virus. Additionally, all or a portion of the adenovirus delayed early gene E3 (which spans mu 76.6 to 86.2) may be eliminated from the helper virus useful in this invention because this gene product is not necessary for the formation of a functioning hybrid virus particle.

It should be noted that one of skill in the art may design other helper viruses or develop other packaging cell lines to complement the adenovirus deletions in the vector construct and enable production of the hybrid virus particle, given this information. Therefore, this invention is not limited by the use or description of any particular helper virus or packaging c ll line.

Thus, as described in Figs. 1A through 1C, the circular plasmid pAd.AV.CMV*LacZ* [SEQ ID NO: 1] (containing the minigene and only adenovirus sequences

from map unit 0 tc 1 and 9 to 16) was digested and co-transfected with a selected Ad5 helper virus (containing adenovirus sequences 9 to 78.4 and 86 to 100) into 293 cells. Thus, the packaging cell line provides the E1 proteins and the helper virus provides all necessary adenovirus gene sequences subsequent to map unit 16. Homologous recombination occurs between the helper virus and the hybrid vector, resulting in the hybrid viral particle. Growth of this hybrid viral particle in 293 cells has been closely monitored for greater than 20 rounds of amplification with no indication of genome instability. Rescue and integration of the transgene from the hybrid virus into a host cell and further modifications of the vector are described below. The resulting hybrid virus Ad.AV.CMV*LacZ* combines the high titer potential of adenovirus with the integrating biology associated with AAV latency.

G. Hybrid Virus Polycation Conjugates

Rep expression is required for rescue of the rAAV genome to occur. A preferred approach is to synthetically incorporate a plasmid permitting expression of *rep* into the hybrid particle. To do so, the hybrid viruses described above are further modified by resort to adenovirus-polylysine conjugate technology. See, e.g., Wu et al, J. Biol. Chem., 264:16985-16987 (1989); and K. J. Fisher and J. M. Wilson, Biochem. J., 299: 49 (April 1, 1994), incorporated herein by reference. Using this technology, a hybrid virus as described above is modified by the addition of a poly-cation sequence distributed around the capsid of the hybrid viral particle. Preferably, the poly-cation is poly-lysine, which attaches around th negatively-charged virus to form an external positive charg . A plasmid containing the AAV *rep* gene (or a functional portion th reof) under the control of a suitable promoter is then complexed directly

to the hybrid capsid, resulting in a single viral particle containing the hybrid virus and an AAV *rep* gene. The negatively charged plasmid DNA binds with high affinity to the positively charged polylysine. Essentially the techniques employed in constructing this hybrid virus conjugate or trans-infection particle are as described in detail in Example 3 below.

An alternative embodiment of the hybrid vector and resulting viral particle is provided by altering the *rep* containing plasmid to also contain an AAV *cap* gene. This embodiment of the hybrid vector when in a host cell is thus able to produce a recombinant AAV particle, as discussed in more detail below.

The plasmids employed in these embodiments contain conventional plasmid sequences, which place a selected AAV sequence, i.e., *rep* and/or *cap* gene sequences, under the control of a selected promoter. In the example provided below, the exemplary plasmid is pRep78/52 [SEQ ID NO: 2], a trans-acting plasmid containing the AAV sequences that encode *rep* 78 kD and 52 kD proteins under the control of the AAV P5 promoter. The plasmid also contains an SV40 polyadenylation signal. The DNA sequence of this plasmid is provided in Fig. 8 [SEQ ID NO: 2].

In a similar manner and with resort to plasmid and vector sequences known to the art, analogous plasmids may be designed using both *rep* and *cap* genes, and different constitutive or regulated promoters, optional poly-A sequences and introns.

The availability of materials to make these modified hybrid vectors and viruses and the AAV *rep* and/or *cap* containing vectors and the techniques involved in the assembly of th hybrid vector and *rep* and/ r *cap* containing plasmids ar conventi nal as described above. The assembly techniques for the trans-infection particle

employ the techniques described above for the hybrid vector and the techniques of Wu et al and Fisher et al, cited above. The use of this trans-infection particle including rescue and integration of the transgene into the host cell is described below.

II. Function of the Hybrid Virus

A. *The Hybrid Virus Infects a Target Cell*

Once the hybrid virus or trans-infection particle is constructed as discussed above, it is targeted to, and taken up by, a selected target cell. The selection of the target cell also depends upon the use of the hybrid virus, i.e., whether or not the transgene is to be replicated *in vitro* for production of a recombinant AAV particle, or *ex vivo* for production into a desired cell type for redelivery into a patient, or *in vivo* for delivery to a particular cell type or tissue. Target cells may therefor be any mammalian cell (preferably a human cell). For example, in *in vivo* use, the hybrid virus can target to any cell type normally infected by adenovirus, depending upon the route of administration, i.e., it can target, without limitation, neurons, hepatocytes, epithelial cells and the like. Uptake of the hybrid virus by the cell is caused by the infective ability contributed to the vector by the adenovirus and AAV sequences.

B. *The Transgene is Rescued.*

Once the hybrid virus or trans-infection particle is taken up by a cell, the AAV ITR flanked transgene must be rescued from the parental adenovirus backbone. Rescue of the transgene is dependent upon supplying the infected cell with an AAV *rep* gene. Thus, efficacy of the hybrid virus can be measured in terms of *rep* mediated rescue f rAAV from the parental adenovirus template.

The *rep* genes can be supplied to the hybrid virus by several methods. One embodiment for providing *rep* proteins in *trans* was demonstrated with the exemplary hybrid virus Ad.AV.CMV*LacZ* by transfecting into the target monolayer of cells previously infected with the hybrid vector, a liposome enveloped plasmid pRep78/52 [SEQ ID NO: 2] containing the genes encoding the AAV *rep* 78 kDa and 52 kDa proteins under the control of the AAV P5 promoter. Rescue and amplification of a double-stranded AAV monomer and a double-stranded AAV dimer, each containing the *LacZ* transgene described above, was observed in 293 cells. This is described in detail in Example 2.

The production of *rep* in *trans* can be modulated by the choice of promoter in the *rep* containing plasmid. If high levels of *rep* expression are important early for rescue of the recombinant AAV domain, a heterologous (non-adenovirus, non-AAV) promoter may be employed to drive expression of *rep* and eliminate the need for E1 proteins. Alternatively, the low levels of *rep* expression from P5 that occur in the absence of adenovirus E1 proteins may be sufficient to initiate rescue and optimal to drive integration of the recombinant AAV genome in a selected use.

More preferably for *in vivo* use, the AAV *rep* gene may also be delivered as part of the hybrid virus. One embodiment of this single particle concept is the polycation conjugated hybrid virus (see Fig. 7). Infection of this trans-infection particle is accomplished in the same manner and with regard to the same target cells as identified above. The polylysine conjugate of the hybrid virus onto which was directly complexed a plasmid that encoded the *rep* 78 and 52 proteins, combines all of the functional components into a single particle structure. Thus, th trans-infection

particle permits delivery of a single particle to the cell, which is considerably more desirable for therapeutic use. Similar experiments to demonstrate rescue of the transgene from the hybrid conjugate trans-infection particle in 293 cells and in HeLa cells are detailed in Example 4.

In another embodiment, the hybrid virus is modified by cloning the *rep* cDNA directly into the adenovirus genome portion of the hybrid vector. Because it is known that even residual levels of *rep* expression can interfere with replication of adenovirus DNA, such incorporation of *rep* into the hybrid vector itself is anticipated to requires possible mutation of the *rep* genes to encode only selected domains, and the use of inducible promoters to regulate *rep* expression, as well as careful placement of the *rep* genes into the adenovirus sequences of the hybrid vector.

c. *Transgene Integrates into Chromosome*

Once uncoupled (rescued) from the genome of the hybrid virus, the recombinant AAV/transgene minigene seeks an integration site in the host chromatin and becomes integrated therein, providing stable expression of the accompanying transgene in the host cell. This aspect of the function of the hybrid virus is important for its use in gene therapy. The AAV/transgene minigene sequence rescued from the hybrid virus achieves provirus status in the target cell, i.e., the final event in the hybrid lifecycle (Fig. 7).

To determine whether the AAV minigene rescued from the hybrid virus achieves provirus status in a target cell, non-E1 expressing HeLa cells were infected with th hybrid vector-poly-Lysine conjugate complexed with pR p78/52 [SEQ ID NO: 2] and passaged until stable colonies of *LacZ* expressing cells are evident. A duplicate plate of cells was inf cted with the sam

conjugate, but instead of being complexed with the pRep78/52 plasmid [SEQ ID NO: 2], carried an irrelevant plasmid. Cells that receive the *rep* containing hybrid particle produced a greater number of stable *LacZ* positive colonies than cells infected with the control vector. This indicates multiple rescue and integration events in cells that expressed *rep* proteins. Confirmation of integration is revealed by characterizing the recombinant AAV genome in the hybrid infected cells and identifying flanking chromosomal sequences (see Example 5).

III. Use of the Hybrid Viruses and Viral Particles in Gene Therapy

The novel hybrid virus and trans-infection particles of this invention provide efficient gene transfer vehicles for somatic gene therapy. These hybrid viruses are prepared to contain a therapeutic gene in place of the *LacZ* reporter transgene illustrated in the exemplary vector. By use of the hybrid viruses and trans-infection particles containing therapeutic transgenes, these transgenes can be delivered to a patient *in vivo* or *ex vivo* to provide for integration of the desired gene into a target cell. Thus, these hybrid viruses and trans-infection particles can be employed to correct genetic deficiencies or defects. Two examples of the generation of gene transfer vehicles for the treatment of cystic fibrosis and familial hypercholesterolemia are described in Examples 6 and 7 below. One of skill in the art can generate any number of other gene transfer vehicles by including a selected transgene for the treatment of other disorders. For example, the trans-infection particles are anticipated to be particularly advantageous in *ex vivo* gene therapy

where transduction and proviral integration in a stem cell is desired, such as in bone marrow directed gene therapy.

The hybrid viruses and trans-infection particles of the present invention may be administered to a patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

The hybrid viruses and trans-infection particles of this invention may be administered in sufficient amounts to transfect the desired cells and provide sufficient levels of integration and expression of the selected transgene to provide a therapeutic benefit without undue adverse or with medically acceptable physiological effects which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of administration include direct delivery to the target organ, tissue or site, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other parental routes of administration. Routes of administration may be combined, if desired.

Dosages of the hybrid virus and/or trans-infection particle will depend primarily on factors such as the condition being treated, the selected gene, the age, weight and health of the patient, and may thus vary among patients. A therapeutically effective human dose of the hybrid viruses or trans-infection particles of the present invention is believed to be in the range of from about 20 to about 50 ml of saline solution containing

concentrations of from about 1 x 10^7 to 1 x 10^{10} pfu/ml hybrid virus of the present invention. A preferred human dose is about 20 ml saline solution at the above concentrations. The dosage will be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the selected gene can be monitored to determine the selection, adjustment or frequency of dosage administration.

IV. High Efficiency Production of rAAV

The hybrid viruses and trans-infection particles of this invention have another desirable utility in the production of large quantities of recombinant AAV particles. Due to the complicated current methods for generating AAV, there is only a limited amount of AAV available for use in industrial, medical and academic biotechnology procedures. The vectors and viruses of the present invention provide a convenient and efficient method for generating large quantities of rAAV particles.

According to this aspect of the invention, a trans-infection particle is constructed as described above and in Example 3 and is employed to produce high levels of rAAV as detailed in Example 8, with the possible modifications described in Example 9 below. Briefly, a plasmid is generated that contains both AAV *rep* and *cap* genes under the control of a suitable plasmid and is complexed to the poly-lysine exterior of the hybrid virus as described above. This trans-infection particle is then permitted to infect a selected host cell, such as 293 cells. The presence of both *rep* and *cap* permit th formati n of AAV particles in the cells and generat an AAV virus titer of about 10^9 virions. In contrast, current meth ds involving the transfection of multiple plasmids produce only about 10^7 virion tit r.

The rAAV is isolated from the culture by selecting the *LacZ*-containing blue plaques and purifying them on a cesium chloride gradient.

The benefit of this procedure relates to the fact that the *cis* AAV element is encoded by the parental adenovirus genome. As a result, the *trans* plasmid is the only DNA component that is needed for complex formation. The cell is thereby loaded with significantly more copies of the trans-acting *rep* and *cap* sequences, resulting in improved efficiency of rescue and packaging.

Numerous comparative studies focusing on the optimal ratio and copy number of the *cis* and *trans* plasmids for AAV production indicated that there is a positive correlation between the *trans* plasmid copy number and yield of recombinant virus. As described in detail in Example 8, the yield of recombinant AV.CMV*LacZ* virus was increased by 5-10 fold by using the trans-infection particle instead of a standard adenovirus vector.

The primary limitation associated with the production of recombinant AAV using a hybrid virus of this invention relates to difficulties that arise in distinguishing between the two viruses (i.e., adenovirus and AAV) that are produced by the cell. Using the exemplary vectors and vector components of this invention, *LacZ* histochemical staining could not be used to titer the yield of recombinant AV.CMV*LacZ* since any contaminating Ad.AV.CMV*LacZ* hybrid would contribute to the final count. Therefore, a rapid Southern blot technique for quantitating yields of recombinant AAV was incorporated. The assay that was developed enabled not only quantitation and verificati n of AAV production, but also demonstrated the r moval f contaminating hybrid virus from purified AAV stocks.

Another method for detecting contaminating hybrid virions involves modifying the hybrid vector by inserting a small second reporter minigene (i.e., reporter gene, promoter and other expression control sequences, where desired) into the E3 region of the parental adenovirus backbone. Because this reporter is not linked to the AAV domain, contaminating hybrid virus that is present during purification can be monitored by this hybrid-specific marker. Another possible reporter gene is the nucleic acid sequence for green fluorescent protein. With this hybrid vector containing two reporter sequences, histochemical staining for alkaline phosphatase (adenovirus reporter) or β-galactosidase (AAV reporter) activity can be used to monitor each viral domain.

The following examples illustrate the construction and testing of the hybrid vectors of the present invention and the use thereof in the productions of recombinant AAV. These examples are illustrative only, and do not limit the scope of the present invention.

Example 1 - Construction of a Hybrid Virus

A first hybrid adenovirus-AAV virus was engineered by homologous recombination between DNA extracted from an adenovirus and a complementing vector according to protocols previously described [see, e.g., K. F. Kozarsky et al, J. Biol. Chem., 269:13695-13702 (1994) and references cited therein]. The following description refers to the diagram of Fig. 1.

Adenovirus DNA was extracted from CsCl purified d17001 virions, an Ad5 (serotype subgroup C) variant that carries a 3 kb deletion between mu 78.4 through 86 in the nonessential E3 r gion (provided by Dr. William Wold, Washington University, St. Louis, Missouri). Adenoviral

DNA was prepared for co-transfection by digestion with ClaI (adenovirus genomic bp position 917) which removes the left arm of the genome encompassing adenovirus map units 0-2.5. See lower diagram of Fig. 1B.

The complementing hybrid vector, pAd.AV.CMV*LacZ* (see Fig. 1A and Fig. 2 [SEQ ID NO: 1]) was constructed as follows:

A parental cloning vector, pAd.BglII was designed. It contains two segments of wild-type Ad5 genome (i.e., map units 0-1 and 9-16.1) separated by a unique BglII cloning site for insertion of heterologous sequences. The missing Ad5 sequences between the two domains (adenovirus genome bp 361-3327) results in the deletion of E1a and the majority of E1b following recombination with viral DNA.

A recombinant AAV genome (AV.CMV*LacZ*) was designed and inserted into the BglII site of pAd.BglII to generate the complementing plasmid. The linear arrangement of AV.CMV*LacZ* [SEQ ID NO: 1] (see top diagram of Fig. 1B) includes:

(a) the 5' AAV ITR (bp 1-173) obtained by PCR using pAV2 [C. A. Laughlin et al, Gene, 23: 65-73 (1983)] as template [nucleotide numbers 365-538 of Fig. 2 [SEQ ID NO: 1]];

(b) a CMV immediate early enhancer/promoter [Boshart et al, Cell, 41:521-530 (1985); nucleotide numbers 563-1157 of Fig. 2 [SEQ ID NO: 1]],

(c) an SV40 splice donor-splice acceptor (nucleotide numbers 1178-1179 of Fig. 2 [SEQ ID NO: 1]),

(d) *E. coli* beta-galactosidase cDNA (nucleotide numbers 1356 - 4827 of Fig. 2 [SEQ ID NO: 1]),

(e) an SV40 polyadenylation signal (a 237 Bam HI-BclI restriction fragment containing the cleavage/poly-A signals from both the early and late

transcription units; nucleotide numbers 4839 - 5037 of Fig. 2 [SEQ ID NO: 1]) and

(f) 3'AAV ITR, obtained from pAV2 as a SnaBI-BglII fragment (nucleotide numbers 5053 - 5221 of Fig. 2 [SEQ ID NO: 1]).

The resulting complementing hybrid vector, pAd.AV.CMV*LacZ* (see Fig. 1A and Fig. 2 [SEQ ID NO: 1]), contained a single copy of recombinant AV.CMV*LacZ* flanked by adenovirus coordinates 0-1 on one side and 9-16.1 on the other. Plasmid DNA was linearized using a unique NheI site immediately 5' to adenovirus map unit zero (0) (resulting in the top diagram of Fig. 1B).

Both the adenovirus substrate and the complementing vector DNAs were transfected to 293 cells [ATCC CRL1573] using a standard calcium phosphate transfection procedure [see, e.g., Sambrook et al, cited above]. The end result of homologous recombination involving sequences that map to adenovirus map units 9-16.1 is hybrid Ad.AV.CMV*LacZ* (see Fig. 1C) in which the E1a and E1b coding regions from the d17001 adenovirus substrate are replaced with the AV.CMV*LacZ* from the hybrid vector.

Twenty-four hours later, the transfection cocktail was removed and the cells overlayed with 0.8% agarose containing 1x BME and 2% fetal bovine serum (FBS). Once viral plaques developed (typically 10-12 days post-transfection), plaques were initially screened for *E. coli* β-galactosidase (*LacZ*) activity by overlaying the infected monolayer with agarose supplemented with a histochemical stain for *LacZ*, according to the procedure described in J. Price et al, Proc. Natl. Acad. Sci., USA, 84:156-160 (1987). Positive cl nes (identified by the deposit of insoluble blue dye) were isolated, subjected to three rounds of fre ze (dry ice/ethanol)- thaw (37°C) and an aliquot f the suspended plaque was used to infect

a fresh monolayer of 293 cells seeded on duplicate 60mm plates.

Twenty-four hours later the cells from one set of plates were fixed and again stained for *LacZ* activity. Cells from the duplicate plate were harvested, suspended in 0.5 ml 10 mM Tris-Cl, pH8.0, and lysed by performing a series of three freeze (dry ice/ethanol)-thaw (37°C) cycles. Cell debris was removed by centrifugation and an aliquot of the supernatant used to measure *LacZ* enzyme activity.

As indicated in Fig. 3, assays for β-galactosidase activity which measured the absorbance at 420 nm of the beta-galactosidase blue color in successful recombinants, revealed that eight of the ten isolated, putative positive clones (D1A through D1J) expressed high levels of enzyme. Histochemical staining produced similar results.

Large-scale production and purification of recombinant virus was performed as described in Kozarsky et al, cited above, and references cited therein.

Example 2 - Functional Analysis of Hybrid Vector

The ability to rescue the AV.CMV*LacZ* sequence [SEQ ID NO: 1] from the hybrid virus represented an important feature of the hybrid vector and virus systems of Example 1. To evaluate this feature, it was necessary to provide the necessary AAV gene products in *trans* that direct AAV excision and amplification (i.e. *rep* proteins). Furthermore, this experiment was conducted in 293 cells to transcomplement the E1 deletion in the Ad.AV.CMV*LacZ* clones, because the adenovirus E1 gene proteins have been shown to be important for initiating the lytic phase of the AAV lif cycle.

293 cells were seeded onto 6-w 11 35 mm plates at a density of 1 x 10^6 cells/well. Twenty-four hours

later, seeding media [DMEM/10% FBS supplemented with antibiotics] was replaced with 1.0 ml DMEM/2% FBS and infected with Ad.AV.CMV*LacZ* hybrid clones at an MOI of 1. Two hours later, each well was transfected with 1 µg plasmid pRep78/52 [SEQ ID NO: 2], a trans-acting plasmid that encodes the sequence encoding the AAV *rep* 78 kD and 52 kD proteins. The *rep* sequences in this construct are under the control of the AAV P5 promoter and utilize an SV40 polyadenylation signal.

As a positive control for AAV rescue, 293 cells seeded in a 6-well plate as above were co-transfected with a cis-acting AAV plasmid pAV.CMV*LacZ* and pRep78/52. pAV.CMV*LacZ* contained AV.CMV*LacZ*, the identical sequence encoded by pAd.AV.CMV*LacZ* [SEQ ID NO: 1] described in Example 1 cloned into the BglII site of pSP72 (Promega).

To provide the necessary adenovirus helper function for AAV rescue, cells were infected with either wild-type Ad5 virus or a first generation E1-deleted virus Ad.CMhpAP at an MOI of 5, approximately 2 hours prior to adding the transfection cocktail. Ad.CMhpAP is identical to Ad.CMV*LacZ* (Example 1) with the modification that the alkaline phosphatase sequence (which can be obtained from Genbank) is inserted in place of the *LacZ* gene.

Transfections were performed with Lipofectamine (Life Technologies) according to the instructions provided by the manufacturer. Thirty hours post-transfection, the cells were harvested and episomal DNA (Hirt extract) prepared as described by J. M. Wilson et al, J. Biol. Chem., 267:(16):11483-11489 (1992). Samples were resolved on a 1.2% agarose gel and electroblotted onto a nylon membrane. Blots w re hybridized (Southern) with a ^{32}P random prim r-label d r striction fragment isolated from the *E. coli LacZ* cDNA.

The full spectrum of duplex molecular species that appear during a lytic AAV infection (i.e., monomeric forms of the double stranded intermediates, RFm and RFd, respectively) were evident in transfected cells infected with wild type and E1 deleted Ad5. No replicative intermediates were detected when transfections were performed in the absence of helper virus.

Hirt extracts from the 293 cells infected with putative Ad.AV.CMV*LacZ* hybrid clones D1A and D1C revealed a single band corresponding to the viral DNA, when probed with a *LacZ* restriction fragment. In the presence of *rep* proteins 78 and 52, however, the same clones yielded a banding pattern that included not only the adenovirus DNA, but an RF monomer and dimer of AV.CMV*LacZ*. A single-stranded form of AV.CMV*LacZ* [SEQ ID NO: 1] was not evident. Two additional clones gave similar banding patterns, D1B and D1H. In all, each of the eight Ad.AV.CMV*LacZ* hybrids that were found in Fig. 3 to express high levels of *Lac Z* activity were positive for rescue of the AAV domain.

With the exception of an extra band of approximately 3.5 kb, the rescue of the AV.CMV*LacZ* [SEQ ID NO: 1] from the hybrid viral DNA was nearly identical to results obtained from a standard *cis* and *trans* plasmid-based approach. In these later samples, adenovirus helper function was provided by pre-infecting cells with either wild-type Ad5 or an E1-deleted recombinant virus Ad.CBhpAP (also termed H5.CBALP). The Ad.CBhpAP virus has the same sequence as the Ad.CMhpAP virus described above, except that the CMV promoter sequence is replaced by the chicken cytoplasmic ß-actin promoter [nucleotides +1 to +275 as described in T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)]. The level of rescue in c lls infected with WT Ad5 appeared to be greater relative to those infected with the

recombinant Ad.CBhpAP virus, likely due to the additional E1 expression provided by the wild-type genome. The relevance of including an E1 deleted adenovirus here is to document that the level of adenovirus E1 proteins expressed in 293 cells is sufficient for AAV helper function.

Example 3 - Synthesis of Polylysine Conjugates

Another version of the viral particle of this invention is a polylysine conjugate with a *rep* plasmid complexed directly to the hybrid virus capsid. This conjugate permits efficient delivery of the *rep* expression plasmid pRep78/52 [SEQ ID NO: 2] in tandem with the hybrid virus, thereby removing the need for a separate transfection step. See, Fig. 8 for a diagrammatic outline of this construction.

Purified stocks of a large-scale expansion of Ad.AV.CMV*LacZ* clone D1A were modified by coupling poly-L-lysine to the virion capsid essentially as described by K. J. Fisher and J. M. Wilson, Biochem. J., 299:49-58 (1994), resulting in an Ad.AV.CMV*LacZ*-$(Lys)_n$ conjugate. The procedure involves three steps. First, hybrid virions are activated through primary amines on capsid proteins with the heterobifunctional water-soluble cross-linking agent, sulpho-SMCC [sulpho-(N-succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate] (Pierce). The conjugation reaction, which contained 0.5 mg (375 nmol) of sulpho-SMCC and 6 x 10^{12} A_{260} hybrid vector particles in 3.0 ml of HBS, was incubated at 30°C for 45 minutes with constant gentle shaking. This step involved formation of a peptide bond between the active N-hydroxysuccinimide (NHS) ester of sulpho-SMCC and a free amine (e.g. lysine) contributed by an adenovirus protein sequence (capsid prot in) in th recombinant virus, yielding a maleimide-activated viral particle.

Unincorporated, unreacted cross-linker was removed by gel filtration on a 1 cm x 15 cm Bio-Gel P-6DG (Bio-Rad Laboratories) column equilibrated with 50 mM Tris/HCl buffer, pH 7.0, and 150 mM NaCl. Peak A_{260} fractions containing maleimide-activated hybrid virus were combined and placed on ice.

Second, poly-L-lysine having a molecular mass of 58 kDa at 10 mg/ml in 50 mM triethanolamine buffer (pH 8.0), 150 mM NaCl and 1 mM EDTA was thiolated with 2-imminothiolane/HCl (Traut's Reagent; Pierce) to a molar ratio of 2 moles-SH/mole polylysine under N_2; the cyclic thioimidate reacts with the poly(L-lysine) primary amines resulting in a thiolated polycation. After a 45 minute incubation at room temperature the reaction was applied to a 1 cm x 15 cm Bio-Gel P6DG column equilibrated with 50 mM Tris/HCl buffer (pH 7.0), 150 mM NaCl and 2 mM EDTA to remove unincorporated Traut's Reagent.

Quantification of free thiol groups was accomplished with Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)], revealing approximately 2 mol of -SH/mol of poly(L-lysine). The coupling reaction was initiated by adding 1 x 10^{12} A_{260} particles of maleimide-activated hybrid virus/mg of thiolated poly(L-lysine) and incubating the mixture on ice at 4°C for 15 hours under argon. 2-mercaptoethylamine was added at the completion of the reaction and incubation carried out at room temperature for 20 minutes to block unreacted maleimide sites.

Virus-polylysine conjugates, Ad.AV.CMV*LacZ*-$(Lys)_n$, were purified away from unconjugated poly(L-lysine) by ultracentrifugation through a CsCl step gradient with an initial composition of equal volumes of 1.45 g/ml (bottom step) and 1.2 g/ml (top step) CsCl in 10 mM Tris/HCl buffer (pH 8.0). Centrifugation was at 90,000 g for 2 hours at 5°C. The final product was

dialyzed against 20 mM Hepes buffer (pH 7.8) containing 150 mM NaCl (HBS).

Complexes of Ad.AV.CMV*LacZ*-$(Lys)_n$ with pRep78/52 plasmid DNA [SEQ ID NO: 2] were formed by adding varying quantities of Ad.AV.CMV*LacZ*-$(Lys)_n$ in 50µl HBS to 0.5 µg of pRep78/52 plasmid DNA [SEQ ID NO: 2] in 50µl HBS. After 30 minutes incubation at room temperature, a complex was formed of the hybrid virus Ad.AV.CMV*LacZ*-$(Lys)_n$ associated in a single particle with the plasmid DNA containing the *rep* genes.

This complex, termed a trans-infection particle, was evaluated for DNA binding capacity by gel mobility shift assays performed as described in Fisher et al, cited above. This analysis revealed that the plasmid binding capacity of the purified conjugate (expressed as the number of A_{260} particles Ad.AV.CMV*LacZ*-$(Lys)_n$ that can neutralize the charge contributed by 1 µg plasmid DNA) was 1 µg pRep78/52 plasmid DNA/6.0 x 10^{10} A_{260} particles Ad.AV.CMV*LacZ*-$(Lys)_n$.

Example 4 - Trans-Infection Protocol to Demonstrate AAV Excision and Amplification

Trans-infection complexes were prepared by mixing Ad.AV.CMV*LacZ*-$(Lys)_n$ conjugate with pRep78/52 plasmid [SEQ ID NO: 2] and applied to 293 cells as follows. Ad.AV.CMV*LacZ*-$(Lys)_n$ (6 x 10^{10} A_{260} particles) in 100 µl DMEM was added dropwise to a microfuge tube containing 1 µg plasmid DNA in 100 µl DMEM. The mixture was gently mixed and allowed to incubate at room temperature for 10-15 minutes. The trans-infection cocktail was added to 293 cells seeded in a 35 mm 6-well as detailed above. Thirty hours later, cells were harvested and Hirt extracts prepared.

Samples were resolved on a 1.2% agarose gel and electroblotted onto a nylon membrane. Blots were hybridized (Southern) with a P-32 random primer-labeled restriction fragment isolated from the *E. coli LacZ* cDNA.

The Hirt extracts from 293 cells revealed a banding pattern that suggested the AV.CMV*LacZ* minigene sequence [SEQ ID NO: 1] was efficiently rescued from the hybrid conjugate. Both an RF monomer and dimer of the recombinant AV.CMV*LacZ* sequence were evident. As was observed previously, the rescue event was dependent on *rep* proteins since 293 cells that were trans-infected with a hybrid conjugate complexed with an irrelevant reporter plasmid expressing alkaline phosphatase (i.e. pCMVhpAP) revealed only Ad.AV.CMV*LacZ* DNA. This negative control for rescue was secondarily useful for demonstrating the high efficiency of gene transfer to 293 cells that was achieved with the conjugate vehicle.

A duplicate set of 293 cells that received hybrid conjugate which was further complexed with alkaline phosphatase expression plasmid were fixed 24 hours after addition of the trans-infection cocktail and histochemically stained for *LacZ* as described in Price et al, cited above, or for alkaline phosphatase activity as described in J. H. Schreiber et al, BioTechniques, 14:818-823 (1993). Here *LacZ* was a marker for the Ad.AV.CMV*LacZ* hybrid, while alkaline phosphatase served as a reporter for the carrier plasmid. Greater than 90% of the monolayer was transduced with both β-galactosidase and alkaline phosphatase transgenes, showing the high efficiency of the conjugate delivery vehicle (differential staining revealed a blue color for the hybrids containing the *LacZ* marker and a purple color for the plasmids bearing th AP marker).

Because of the important role E1 proteins have for progression of the AAV lifecycle, it was critical to

test the efficiency of the hybrid delivery system in a setting where E1 proteins are not expressed. A trans-infection experiment using the hybrid conjugate complexed with pRep78/52 [SEQ ID NO: 2] was therefore conducted in HeLa cells [ATCC CCL2] to remove the involvement of E1 proteins. The findings suggested rescue of AV.CMV*LacZ* occurred evidenced by the accumulation of RF monomers and dimers. Rescue from HeLa cells (which unlike the 293 cells do not contain any adenovirus E1 proteins) revealed lower levels of rescue of the transgene. The expression of *rep* from the AAV P5 promoter is upregulated by adenovirus E1 and signals the beginning of the AAV lytic cycle. In the absence of E1, *rep* expression from the P5 promoter is virtually silent which is important for maintenance of the proviral latent stages of the AAV lifecycle. It is anticipated that a promoter not dependent on E1 expression will upon substitution for P5, overcome this problem.

Example 5 - Integration of the Transgene

A preliminary study has been performed to determine whether the AAV sequence rescued from the hybrid virus can achieve provirus status in a target cell (Fig. 7). Briefly, HeLa cells [ATCC CCL 2] were infected with the hybrid conjugate complexed with pRep78/52 [SEQ ID NO: 2] and passaged until stable colonies of *LacZ* expressing cells were evident. A duplicate plate of cells was infected with the same conjugate, but instead of being complexed with the pRep78/52 plasmid [SEQ ID NO: 2], carried an irrelevant plasmid. These findings indicated that cells that received the Rep containing hybrid particle produced a greater number of stable *LacZ* positiv colonies than cells that were infected with the control virus. This could be interpreted as a reflection of multiple rescue and integrati n events in cells that

expressed Rep proteins. However, it is possible that an episomal form of AAV that can persist for extended periods of time was present.

To establish the occurrence of integration into the chromosome of the minigene Av.CMV*LacZ* from the hybrid conjugate, the following experiment is performed. The Ad.AV.CMV*LacZ*-$(Lys)_n$ conjugate carrying pRep78/52 plasmid [SEQ ID NO: 2] is used to infect HeLa cells [ATCC CRL2] (primary fibroblasts may also be used). The infected cells are passaged for several generations. The cells are grown to confluency, split and allowed to grow to confluency again, split again and this cycle repeated as desired. This permits sufficient time for uptake, expression, replication and integration to occur. See Fig. 7.

To verify that the recombinant AAV sequence that was rescued from the hybrid genome (step III of Fig. 7) has integrated into a chromosome of the host cell (step IV of Fig. 7), cells are separated by a Fluorescence Activated Cell Sorter (FACS). By this technique, those cells containing a stable integrated copy of the recombinant AV.CMV*LacZ* minigene are separated based on the presence of the β-galactosidase reporter. These cells are tagged with fluorescein-labeled antibodies that recognize the β-Gal protein, and are then separated from non-transduced cells (i.e. those that did not receive a copy of the AAV minigene) by FACS.

DNA is isolated from this purified population of cells and used to construct a genomic library which is screened for individual clones and the sequence verified. If integration occurs, it is documented directly by sequence analysis.

Example 6 - Gene Transfer Vehicle for Cystic Fibrosis

An adenovirus-AAV-CFTR virus constructed by modifying the hybrid Ad.AV.CMV*lacZ* virus described in Example 1 to contain the cystic fibrosis transmembrane regulator (CFTR) gene [J.R. Riordan et al, Science, 245:1066-1073 (1989)] in place of the lacZ gene, using known techniques. One suitable method involves producing a new vector using the techniques described in Example 1. In this new vector the *LacZ* minigene is replaced with the CFTR minigene. For performance of this method vectors bearing the CFTR gene have been previously described and can be readily constructed. This new or reconstructed vector is used to generate a new virus through homologous recombination as described above. The resulting hybrid virus is termed hybrid Ad.AV.CMV*CFTR*. It has the sequence of Fig. 2 [SEQ ID NO: 1], except that the *LacZ* gene is replaced with CFTR. Alternatively, the *LacZ* gene can be removed from the Ad.AV.CMV*LacZ* vector of Example 1 and replaced with the CFTR gene using known techniques.

This virus (or an analogous hybrid virus with a different promoter, regulatory regions, etc.) is useful in gene therapy alone, or preferably, in the form of a conjugate prepared as described in Example 4.

Treatment of cystic fibrosis, utilizing the viruses provided above, is particularly suited for *in vivo*, lung-directed, gene therapy. Airway epithelial cells are the most desirable targets for gene transfer because the pulmonary complications of CF are usually its most morbid and life-limiting. Thus, the hybrid vector of the invention, containing the CFTR gene, is delivered directly into the airway, e.g. by formulating the hybrid virus above, into a preparation which can be inhaled. For example, the hybrid virus or conjugate of the invention containing the CFTR gene, is suspended in 0.25

molar sodium chloride. The virus or conjugate is taken up by respiratory airway cells and the gene is expressed.

Alternatively, the hybrid viruses or conjugates of the invention may be delivered by other suitable means, including site-directed injection of the virus bearing the CFTR gene. In the case of CFTR gene delivery, preferred solutions for bronchial instillation are sterile saline solutions containing in the range of from about 1×10^7 to 1×10^{10} pfu/ml, more particularly, in the range of from about 1×10^8 to 1×10^9 pfu/ml of the recombinant hybrid virus of the present invention.

Other suitable methods for the treatment of cystic fibrosis by use of gene therapy recombinant viruses of this invention may be obtained from the art discussions of other types of gene therapy vehicles for CF. See, for example, U. S. Patent No. 5,240,846, incorporated by reference herein.

Example 7 - Gene Transfer Vehicle for Familial Hypercholesterolemia

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by abnormalities (deficiencies) in the function or expression of LDL receptors [M.S. Brown and J.L. Goldstein, Science, 232(4746):34-37 (1986); J.L. Goldstein and M.S. Brown, "Familial hypercholesterolemia" in Metabolic Basis of Inherited Disease., ed. C.R. Scriver et al, McGraw Hill, New York, pp1215-1250 (1989).] Patients who inherit one abnormal allele have moderate elevations in plasma LDL and suffer premature life-threatening coronary artery disease (CAD). Homozygous patients have severe hypercholesterolemia and life-threatening CAD in childhood.

A hybrid adenovirus-AAV-LDL virus of the invention is constructed by replacing the lacZ gene in the hybrid Ad.AV.CMVlacZ virus of Example 1 with an LDL receptor gene [T. Yamamoto et al, Cell, 39:27-38 (1984)] using known techniques and as described analogously for CF in the preceding example. Vectors bearing the LDL receptor gene can be readily constructed according to this invention. The resulting hybrid vector is termed pAd.AV.CMV*LDL*.

This plasmid or its recombinant virus is useful in gene therapy of FH alone, or preferably, in the form of a viral conjugate prepared as described in Example 4 to substitute a normal LDL gene for the abnormal allele responsible for the gene.

A. Ex Vivo Gene Therapy

Ex vivo gene therapy can be performed by harvesting and establishing a primary culture of hepatocytes from a patient. Known techniques may be used to isolate and transduce the hepatocytes with the above vector(s) bearing the LDL receptor gene(s). For example, techniques of collagenase perfusion developed for rabbit liver can be adapted for human tissue and used in transduction. Following transduction, the hepatocytes are removed from the tissue culture plates and reinfused into the patient using known techniques, e.g. via a catheter placed into the inferior mesenteric vein.

B. In Vivo Gene Therapy

Desirably, the *in vivo* approach to gene therapy, e.g. liver-directed, involves the use of the hybrid viruses and viral conjugates described above. A preferred treatment involves infusing a trans-infection particle of the invention containing LDL into the peripheral circulation of the patient. The patient is then evaluated for change in serum lipids and liver tissues.

The hybrid virus or viral conjugate can be used to infect hepatocytes *in vivo* by direct injection into a peripheral or portal vein (10^7-10^8 pfu/kg) or retrograde into the biliary tract (same dose). This effects gene transfer into the majority of hepatocytes.

Treatments are repeated as necessary, e.g. weekly. Administration of a dose of virus equivalent to an MOI of approximately 20 (i.e. 20 pfu/hepatocyte) is anticipated to lead to high level gene expression in the majority of hepatocytes.

Example 8 - Efficient Production of Recombinant AAV using A Hybrid Virus/Conjugate

The following experiment demonstrated that the AAV genome that was rescued from the Ad.AV.CMV*LacZ* hybrid virus could be packaged into an AAV capsid, provided the *cap* open reading frame was supplied in trans. Thus the viruses of this invention are useful in a production method for recombinant AAV which overcomes the prior art complications that surround the high titer production of recombinant AAV.

A. Trans-Infection Protocol for the Production of rAAV

A trans-infection complex was formed composed of the Ad.AV.CMV*LacZ*-$(Lys)_n$ conjugate described above and a transcomplementing plasmid pAdAAV, which is described in detail in R. J. Samulski et al, J. Virol., 63(9):3822-3828 (1989)]. Briefly, plasmid pAdAAV encodes the entire *rep* and *cap* open reading frames in the absence of AAV ITRs, and has been shown to provide the necessary AAV helper functions for replication and packaging of recombinant AAV sequences.

Ad.AV.CMV*LacZ*-$(Lys)_n$ conjugate (4.5×10^{13} A_{260} particles) in 75 ml DMEM was added dropwise with constant g ntle swirling in 25 ml DMEM containing 750 µg

pAdAAV helper plasmid and incubated at room temperature for 10-15 minutes. The complex was diluted with 450 ml DMEM supplemented with 2% FBS and 20 ml aliquots were added to monolayers of 293 cells seeded on 150 mm plates.

Forty hours post trans-infection, cells were harvested, suspended in 12 ml 10 mM Tris-Cl (pH 8.0), and stored at -80°C.

Because the anticipated outcome was the production of hybrid virus Ad.AV.CMV*LacZ* and a recombinant AAV virion (AV.CMV*LacZ*), both of which carry a functional *LacZ* minigene, it was not possible to use detection of *LacZ* activity as an indicator of AV.CMV*LacZ* production. A novel molecular approach was developed that could be performed in one day and permitted identification of the packaged viral DNAs.

B. Purification of rAAV

Briefly, frozen cell suspensions were subjected to three rounds of freeze-thaw cycles to release recombinant AV.CMV*LacZ* and hybrid Ad.AV.CMV*LacZ*. On completion of the final thaw, bovine pancreatic DNAse (2000 units) and ribonuclease (0.2 mg/ml final concentration) was added and the extract incubated at 37°C for 30 minutes. Cell debris was removed by centrifugation (5000xg for 10 minutes) and the clarified supernatant (15 ml) applied to a 22.5 ml step gradient composed of equal volumes of CsCl at 1.2 g/ml, 1.36 g/ml, and 1.45 g/ml 10mM Tris-Cl, pH8.0. Viral particles were banded at 25,000 rpm in a Beckman SW-28 rotor for 8 hours at 4°C. One ml fractions were collected from the bottom of the tube.

The fractions retrieved from the CsCl gradient of partially purified virus are then digested to release viral DNA from virion capsids as follows. A 5.0µl sample of each fraction was transferred to a microfuge tube containing 20 µl capsid digestion buffer

(50mM Tris-Cl, pH8.0, 1.0mM EDTA, pH8,0, 0.5% SDS, and 1.0 mg/ml Proteinase K). The reaction was incubated at 50°C for 1 hour, allowed to cool to room temperature, diluted with 10 µl milli-Q water, and agarose gel loading dye added.

These fractions are then analyzed by Southern blotting. Samples were resolved on a 1.2% agarose gel, electroblotted onto a nylon membrane. A ^{32}P labeled *LacZ* restriction fragment which was common to both vectors was used as a hybridization probe to locate the migration of viral DNA through the agarose gel. Viral bands were quantitated on a Molecular Dynamics Phosphoimager.

A sample of the extract before CsCl banding was also tested and revealed both hybrid Ad.AV.CMV*LacZ* DNA and double-stranded RF forms (monomers and dimers) of the rescued AV.CMV*LacZ* sequence [SEQ ID NO: 1]. A single-stranded monomer of AV.CMV*LacZ* appeared to be present in the crude extract; however, it was not until the virions were concentrated by buoyant density ultracentrifugation that the single-stranded genome became clearly evident. The single-stranded recombinant genome of the virus was distributed over a range of CsCl densities and revealed a biphasic banding pattern. The two peaks of single-stranded rAAV genome occurred at densities of 1.41 and 1.45 g/ml CsCl, consistent with the reported buoyant densities of wild-type AAV in CsCl [L. M. de la Maza et al, J. Virol., 33:1129-1137 (1980)]. Analysis of the fractions corresponding to the two vector forms revealed the rAAV-1.41 species was several orders of magnitude more active for *lacZ* transduction than the denser rAAV-1.45 g/ml variant. To avoid confusion with contaminating Ad.AAV, samples were heat inactivated (60°C for 30 min) before being added to indicator HeLa cells.

The peak fractions of rAAV-1.41 were combined and purified by equilibrium sedimentation in CsCl to eliminate residual adenovirus particles and concentrate rAAV virions. On the final round of ultracentrifugation, a faint but clearly visible opalescent band was observed in the middle of the gradient tube. Fractions that surrounded the band were evaluated for density, absorbance at 260 nm, and *lacZ* transducing particles. As the band eluted from the gradient tube, a well defined peak of 260 nm absorbing material was recorded, with a maximal absorbance occurring at a density of 1.40 g/ml CsCl. Analysis for *lacZ* transducing particles on HeLa cells revealed a peak of activity that mirrored the absorbance profile. These results indicate rAAV was produced from the hybrid Ad.AAV virus. Furthermore, the titers achieved using the hybrid virus were 5-10 fold elevated compared to more conventional recombinant AAV production schemes (i.e., transfections with cis- and trans-acting plasmids). This represents a significant improvement in rAAV production and indicates that the hybrid is useful for large-scale rAAV production.

All references recited above are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as those modifications permitting optimal use of the hybrid viruses as gene therapy vehicles or production vehicles for recombinant AAV production, are believed to be encompass d in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Hybrid Adenovirus-AAV Vector and Methods of Use Thereof

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10398 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCGCTA GCATCATCAA TAATATACCT TATTTTGGAT TGAAGCCAAT 50

ATGATAATGA GGGGGTGGAG TTTGTGACGT GGCGCGGGGC GTGGGAACGG 100

GGCGGGTGAC GTAGTAGTGT GGCGGAAGTG TGATGTTGCA AGTGTGGCGG 150

AACACATGTA AGCGACGGAT GTGGCAAAAG TGACGTTTTT GGTGTGCGCC 200

GGTGTACACA GGAAGTGACA ATTTTCGCGC GGTTTTAGGC GGATGTTGTA 250

GTAAATTTGG GCGTAACCGA GTAAGATTTG GCCATTTTCG CGGGAAAACT 300

GAATAAGAGG AAGTGAAATC TGAATAATTT TGTGTTACTC ATAGCGCGTA 350

ATATTTGTCT AGGGAGATCT GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC 400

CCGGGCAAAG CCCGGGCGTC GGGCGACCTT TGGTCGCCCG GCCTCAGTGA 450

GCGAGCGAGC GCGCAGAGAG GGAGTGGCCA ACTCCATCAC TAGGGGTTCC 500

TTGTAGTTAA TGATTAACCC GCCATGCTAC TTATCTACAA TTCGAGCTTG 550

CATGCCTGCA GGTCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA 600

CCGCCCAACG ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT 650

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GGTAAACTGC CCACTTGGCA GTACATCAAG TGTATCATAT GCCAAGTACG 750

CCCCCTATTG ACGTCAATGA CGGTAAATGG CCCGCCTGGC ATTATGCCCA 800

GTACATGACC TTATGGGACT TTCCTACTTG GCAGTACATC TACGTATTAG 850

TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT 900

GGATAGCGGT TTGACTCACG GGGATTTCCA AGTCTCCACC CCATTGACGT 950

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GAGGTCTATA TAAGCAGAGC TCGTTTAGTG AACCGTCAGA TCGCCTGGAG 1100

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GGTGGTGGTG CAAATCAAAG AACTGCTCCT CAGTGGATGT TGCCTTTACT 1300

TCTAGGCCTG TACGGAAGTG TTACTTCTGC TCTAAAAGCT GCGGAATTGT 1350

ACCCGCGGCC GCAATTCCCG GGGATCGAAA GAGCCTGCTA AAGCAAAAAA 1400

GAAGTCACCA TGTCGTTTAC TTTGACCAAC AAGAACGTGA TTTTCGTTGC 1450

CGGTCTGGGA GGCATTGGTC TGGACACCAG CAAGGAGCTG CTCAAGCGCG 1500

ATCCCGTCGT TTTACAACGT CGTGACTGGG AAAACCCTGG CGTTACCCAA 1550

CTTAATCGCC TTGCAGCACA TCCCCCTTTC GCCAGCTGGC GTAATAGCGA 1600

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AATGGCGCTT TGCCTGGTTT CCGGCACCAG AAGCGGTGCC GGAAAGCTGG 1700

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TTACGGTCAA TCCGCCGTTT GTTCCCACGG AGAATCCGAC GGGTTGTTAC 1850

TCGCTCACAT TTAATGTTGA TGAAAGCTGG CTACAGGAAG GCCAGACGCG 1900

AATTATTTTT GATGGCGTTA ACTCGGCGTT TCATCTGTGG TGCAACGGGC 1950

GCTGGGTCGG TTACGGCCAG GACAGTCGTT TGCCGTCTGA ATTTGACCTG 2000

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CAGGATATCC TGCTGATGAA GCAGAACAAC TTTAACGCCG TGCGCTGTTC 2650

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TGTATGTGGT GGATGAAGCC AATATTGAAA CCCACGGCAT GGTGCCAATG 2750

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CGCCGGGCAA CTCTGGCTCA CAGTACGCGT AGTGCAACCG AACGCGACCG 3600

CATGGTCAGA AGCCGGGCAC ATCAGCGCCT GGCAGCAGTG GCGTCTGGCG 3650

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TCGAACGCTG GAAGGCGGCG GGCCATTACC AGGCCGAAGC AGCGTTGTTG 3950

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CGCGTGGCAG CATCAGGGGA AAACCTTATT TATCAGCCGG AAAACCTACC 4050

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GGTAGCAGAG CGGGTAAACT GGCTCGGATT AGGGCCGCAA GAAAACTATC 4200

CCGACCGCCT TACTGCCGCC TGTTTTGACC GCTGGGATCT GCCATTGTCA 4250

GACATGTATA CCCCGTACGT CTTCCCGAGC GAAAACGGTC TGCGCTGCGG 4300

GACGCGCGAA TTGAATTATG GCCCACACCA GTGGCGCGGC GACTTCCAGT 4350

TCAACATCAG CCGCTACAGT CAACAGCAAC TGATGGAAAC CAGCCATCGC 4400

CATCTGCTGC ACGCGGAAGA AGGCACATGG CTGAATATCG ACGGTTTCCA 4450

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GTGAGCGAGC GAGCGCGCAG CAGATCTGGA AGGTGCTGAG GTACGATGAG 5250

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4910 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CCCTTTCGTC 4910

WHAT IS CLAIMED IS.

1. A recombinant hybrid virus comprising:

(a) DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(b) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(c) a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(d) DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(e) DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences;

wherein said virus is replication-defective and is provided with a sufficient portion of the genome of the adenovirus to permit infection of the target cell.

2. The virus according to claim 1 wherein said adenovirus is rendered replication defective by a deletion in all or a part of the E1 gene.

3. The virus according to claim 2 wherein said adenovirus genome has a deletion in all or a part of the E3 gene.

4. The virus according to claim 1 wherein said adenovirus genome comprising deletions in the DNA sequences of all or a portion of the adenovirus genes selected from the group consisting of the E2a gene, the E4 gene, the late genes L1 through L5, the intermediate genes IX and IV_a, and a combination thereof.

5. The virus according to claim 1 wherein said selected gene is a reporter gene.

6. The virus according to claim 5 wherein said reporter gene is selected from the group consisting of the genes encoding ß-galactosidase, alkaline phosphatase and green fluorescent protein.

7. The virus according to claim 1 wherein said selected gene is a therapeutic gene.

8. The virus according to claim 7 wherein said therapeutic gene is selected from the group consisting of a normal CFTR gene and a normal LDL gene.

9. The virus according to claim 1 further comprising the DNA of, or corresponding to, a functional portion of the genome of an adeno-associated virus *rep* gene.

10. A recombinant hybrid vector comprising:

(a) DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(b) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(c) a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(d) DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(e) DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences; and

(d) plasmid DNA sequences containing regulatory elements.

11. A recombinant trans-infection particle comprising:

(a) a recombinant hybrid virus comprising:

(i) DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(ii) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(iii) a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(iv) DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(v) DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences;

wherein said virus is replication-defective and is provided with a sufficient portion of the genome of the adenovirus to permit infection of the target cell;

(b) a polycation sequence conjugated to said hybrid virus; and

(c) a plasmid comprising an AAV *rep* gene operatively linked to regulatory sequences capable of directing its expression, said plasmid associated with said polycation sequence.

12. The trans-infection particle according to claim 11 wherein said adenovirus DNA lacks the sequence encoding viral genes.

13. The trans-infection particle according to claim 11 wherein said adenovirus genome is rendered replication-defective by a deletion in all or a part of the E1 gene.

14. The particle according to claim 13 wherein said adenovirus genome has a deletion in all or a part of the E3 gene.

15. The particle according to claim 11 wherein said adenovirus genome has deletions in the DNA sequences of all or a portion of the adenovirus genes selected from the group consisting of the E2a gene, the E4 gene, the late genes L1 through L5, the intermediate genes IX and IV_a, and a combination thereof.

16. The particle according to claim 11 wherein said selected gene is a reporter gene.

17. The particle according to claim 16 wherein said reporter gene is selected from the group consisting of the genes encoding ß-galactosidase, alkaline phosphatase and gr en fluorescent protein.

18. The particle according to claim 11 wherein said selected gene is a therapeutic gene.

19. The particle according to claim 18 wherein said therapeutic gene is selected from the group consisting of a normal CFTR gene and a normal LDL gene.

20. A composition for use in delivering and stably integrating a selected gene into the chromosome of a target cell, said composition comprising

(a) a recombinant hybrid virus comprising:

(i) DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(ii) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(iii) a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(iv) DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(v) DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences;

wherein said virus is replication-defective and is provided with a sufficient portion of the genome of the adenovirus to permit infection of the target cell; and

(b) a pharmaceutically acceptable carrier.

21. The composition according to claim 20 further comprising a plasmid comprising an AAV *rep* gene under the control of regulatory sequences capable of expressing said *rep* gen in said targ t cell.

22. The composition according to claim 21 wherein said vector further comprises the DNA of, or corresponding to, at least a functional portion of the genome of an adeno-associated virus *rep* gene.

23. A composition for use in delivering and stably integrating a selected gene into the chromosome of a target cell comprising an effective amount of a recombinant trans-infection particle in a pharmaceutically acceptable carrier, said particle comprising:

(a) a recombinant hybrid virus comprising:

(i) DNA sequences of, or corresponding to, the 5' inverted terminal repeat (ITR) sequences of an adenovirus and the 5' adenovirus packaging/enhancer domain;

(ii) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) ITR sequences;

(iii) a gene encoding a selected protein operatively linked to regulatory sequences directing expression of the protein in a target cell *in vivo* or *in vitro*;

(iv) DNA sequences of, or corresponding to, the 3' AAV ITR sequences;

(v) DNA sequences of, or corresponding to, the 3' adenovirus ITR sequences;

wherein said virus is replication-defective and is provided with a sufficient portion of the genome of the adenovirus to permit infection of the target cell;

(b) a polycation sequence conjugated to said hybrid virus;

(c) a plasmid comprising an AAV *rep* gene operatively linked to regulatory sequences capable of directing its expression, said plasmid associated with said polycation sequence.

24. A mammalian cell capable of expressing a selected gene introduced therein through transduction of the virus of claim 1, the vector of claim 10, or the trans-infection particle of claim 11.

25. A method for producing high levels of a recombinant adeno-associated virus comprising the steps of

(a) culturing a cell co-transfected with the vector of claim 10 and an optional helper virus in the presence of a plasmid containing an AAV *rep* gene under the control of regulatory sequences capable of expressing said *rep* gene; and

(b) isolating from said culture a recombinant AAV.

26. A method for producing high levels of a recombinant adeno-associated virus comprising the steps of

(a) culturing a cell transfected with the particle of claim 11 and

(b) isolating from said culture a recombinant AAV.

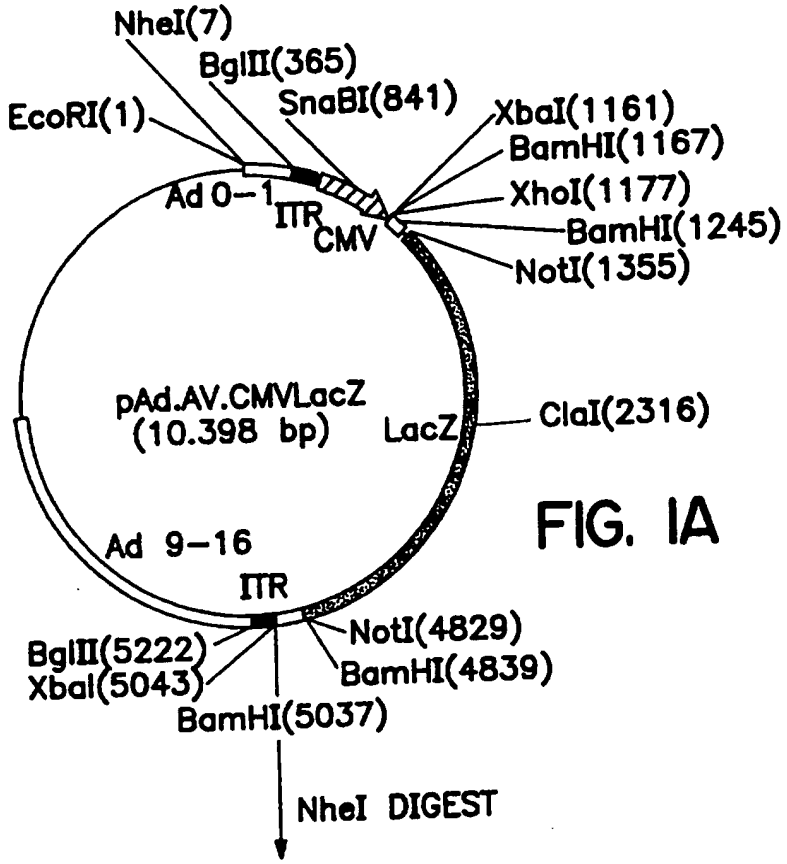


FIG. IA

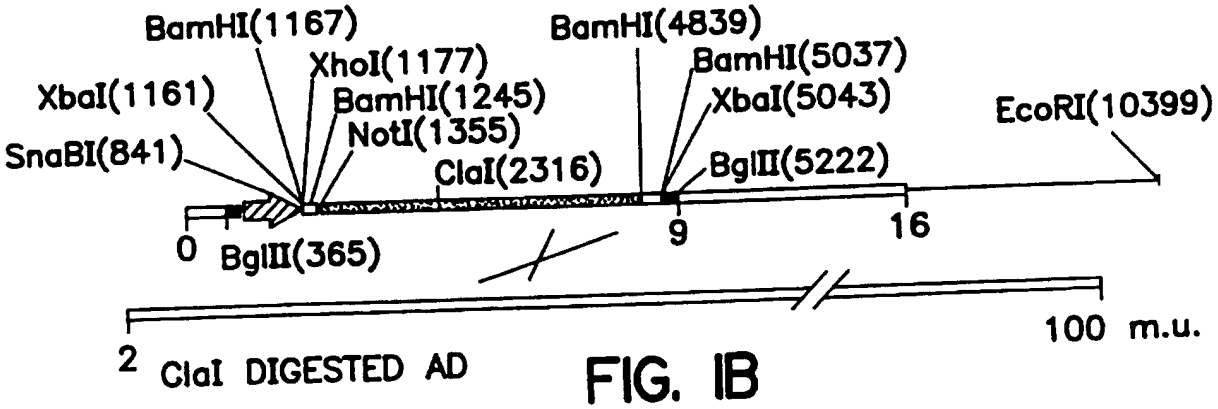


FIG. IB

CO–TRANSFECTION INTO
293 CELLS FOLLOWED BY
INTRACELLULAR HOMOLOGOUS
RECOMBINATION.

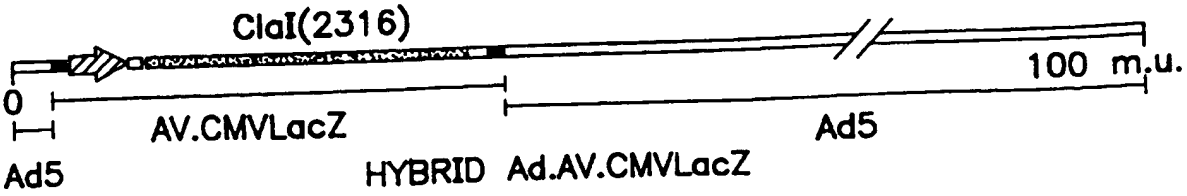


FIG. IC

FIGURE 2A

GAATTCGCTA GCATCATCAA TAATATACCT TATTTTGGAT TGAAGCCAAT ATGATAATGA
60

GGGGGTGGAG TTTGTGACGT GGCGCGGGGC GTGGGAACGG GGCGGGTGAC GTAGTAGTGT
120

GGCGGAAGTG TGATGTTGCA AGTGTGGCGG AACACATGTA AGCGACGGAT GTGGCAAAAG
180

TGACGTTTTT GGTGTGCGCC GGTGTACACA GGAAGTGACA ATTTTCGCGC GGTTTTAGGC
240

GGATGTTGTA GTAAATTTGG GCGTAACCGA GTAAGATTTG GCCATTTTCG CGGGAAAACT
300

GAATAAGAGG AAGTGAAATC TGAATAATTT TGTGTTACTC ATAGCGCGTA ATATTTGTCT
360

AGGGAGATCT GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC CCGGGCAAAG CCCGGGCGTC
420

GGGCGACCTT TGGTCGCCCG GCCTCAGTGA GCGAGCGAGC GCGCAGAGAG GGAGTGGCCA
480

ACTCCATCAC TAGGGGTTCC TTGTAGTTAA TGATTAACCC GCCATGCTAC TTATCTACAA
540

TTCGAGCTTG CATGCCTGCA GGTCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA
600

CCGCCCAACG ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT AGTAACGCCA
660

ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTTAC GGTAAACTGC CCACTTGGCA
720

GTACATCAAG TGTATCATAT GCCAAGTACG CCCCCTATTG ACGTCAATGA CGGTAAATGG
780

CCCGCCTGGC ATTATGCCCA GTACATGACC TTATGGGACT TTCCTACTTG GCAGTACATC
840

TACGTATTAG TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT
900

GGATAGCGGT TTGACTCACG GGGATTTCCA AGTCTCCACC CCATTGACGT CAATGGGAGT
960

TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAAATGTC GTAACAACTC CGCCCCATTG
1020

FIGURE 2B

ACGCAAATGG GCGGTAGGCG TGTACGGTGG GAGGTCTATA TAAGCAGAGC TCGTTTAGTG 1080

AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTTG ACCTCCATAG AAGACACCGG 1140

GACCGATCCA GCCTCCGGAC TCTAGAGGAT CCGGTACTCG AGGAACTGAA AAACCAGAAA 1200

GTTAACTGGT AAGTTTAGTC TTTTTGTCTT TTATTTCAGG TCCCGGATCC GGTGGTGGTG 1260

CAAATCAAAG AACTGCTCCT CAGTGGATGT TGCCTTTACT TCTAGGCCTG TACGGAAGTG 1320

TTACTTCTGC TCTAAAAGCT GCGGAATTGT ACCCGCGGCC GCAATTCCCG GGGATCGAAA 1380

GAGCCTGCTA AAGCAAAAAA GAAGTCACCA TGTCGTTTAC TTTGACCAAC AAGAACGTGA 1440

TTTTCGTTGC CGGTCTGGGA GGCATTGGTC TGGACACCAG CAAGGAGCTG CTCAAGCGCG 1500

ATCCCGTCGT TTTACAACGT CGTGACTGGG AAAACCCTGG CGTTACCCAA CTTAATCGCC 1560

TTGCAGCACA TCCCCCTTTC GCCAGCTGGC GTAATAGCGA AGAGGCCCGC ACCGATCGCC 1620

CTTCCCAACA GTTGCGCAGC CTGAATGGCG AATGGCGCTT TGCCTGGTTT CCGGCACCAG 1680

AAGCGGTGCC GGAAAGCTGG CTGGAGTGCG ATCTTCCTGA GGCCGATACT GTCGTCGTCC 1740

CCTCAAACTG GCAGATGCAC GGTTACGATG CGCCCATCTA CACCAACGTA ACCTATCCCA 1800

TTACGGTCAA TCCGCCGTTT GTTCCCACGG AGAATCCGAC GGGTTGTTAC TCGCTCACAT 1860

TTAATGTTGA TGAAAGCTGG CTACAGGAAG GCCAGACGCG AATTATTTTT GATGGCGTTA 1920

ACTCGGCGTT TCATCTGTGG TGCAACGGGC GCTGGGTCGG TTACGGCCAG GACAGTCGTT 1980

TGCCGTCTGA ATTTGACCTG AGCGCATTTT TACGCGCCGG AGAAAACCGC CTCGCGGTGA 2040

FIGURE 2C

TGGTGCTGCG TTGGAGTGAC GGCAGTTATC TGGAAGATCA GGATATGTGG CGGATGAGCG 2100

GCATTTTCCG TGACGTCTCG TTGCTGCATA AACCGACTAC ACAAATCAGC GATTTCCATG 2160

TTGCCACTCG CTTTAATGAT GATTTCAGCC GCGCTGTACT GGAGGCTGAA GTTCAGATGT 2220

GCGGCGAGTT GCGTGACTAC CTACGGGTAA CAGTTTCTTT ATGGCAGGGT GAAACGCAGG 2280

TCGCCAGCGG CACCGCGCCT TTCGGCGGTG AAATTATCGA TGAGCGTGGT GGTTATGCCG 2340

ATCGCGTCAC ACTACGTCTG AACGTCGAAA ACCCGAAACT GTGGAGCGCC GAAATCCCGA 2400

ATCTCTATCG TGCGGTGGTT GAACTGCACA CCGCCGACGG CACGCTGATT GAAGCAGAAG 2460

CCTGCGATGT CGGTTTCCGC GAGGTGCGGA TTGAAAATGG TCTGCTGCTG CTGAACGGCA 2520

AGCCGTTGCT GATTCGAGGC GTTAACCGTC ACGAGCATCA TCCTCTGCAT GGTCAGGTCA 2580

TGGATGAGCA GACGATGGTG CAGGATATCC TGCTGATGAA GCAGAACAAC TTTAACGCCG 2640

TGCGCTGTTC GCATTATCCG AACCATCCGC TGTGGTACAC GCTGTGCGAC CGCTACGGCC 2700

TGTATGTGGT GGATGAAGCC AATATTGAAA CCCACGGCAT GGTGCCAATG AATCGTCTGA 2760

CCGATGATCC GCGCTGGCTA CCGGCGATGA GCGAACGCGT AACGCGAATG GTGCAGCGCG 2820

ATCGTAATCA CCCGAGTGTG ATCATCTGGT CGCTGGGGAA TGAATCAGGC CACGGCGCTA 2880

ATCACGACGC GCTGTATCGC TGGATCAAAT CTGTCGATCC TTCCCGCCCG GTGCAGTATG 2940

AAGGCGGCGG AGCCGACACC ACGGCCACCG ATATTATTTG CCCGATGTAC GCGCGCGTGG 3000

ATGAAGACCA GCCCTTCCCG GCTGTGCCGA AATGGTCCAT CAAAAAATGG CTTTCGCTAC 3060

FIGURE 2D

CTGGAGAGAC GCGCCCGCTG ATCCTTTGCG AATACGCCCA CGCGATGGGT AACAGTCTTG 3120

GCGGTTTCGC TAAATACTGG CAGGCGTTTC GTCAGTATCC CCGTTTACAG GGCGGCTTCG 3180

TCTGGGACTG GGTGGATCAG TCGCTGATTA AATATGATGA AAACGGCAAC CCGTGGTCGG 3240

CTTACGGCGG TGATTTTGGC GATACGCCGA ACGATCGCCA GTTCTGTATG AACGGTCTGG 3300

TCTTTGCCGA CCGCACGCCG CATCCAGCGC TGACGGAAGC AAAACACCAG CAGCAGTTTT 3360

TCCAGTTCCG TTTATCCGGG CAAACCATCG AAGTGACCAG CGAATACCTG TTCCGTCATA 3420

GCGATAACGA GCTCCTGCAC TGGATGGTGG CGCTGGATGG TAAGCCGCTG GCAAGCGGTG 3480

AAGTGCCTCT GGATGTCGCT CCACAAGGTA AACAGTTGAT TGAACTGCCT GAACTACCGC 3540

AGCCGGAGAG CGCCGGGCAA CTCTGGCTCA CAGTACGCGT AGTGCAACCG AACGCGACCG 3600

CATGGTCAGA AGCCGGGCAC ATCAGCGCCT GGCAGCAGTG GCGTCTGGCG GAAAACCTCA 3660

GTGTGACGCT CCCCGCCGCG TCCCACGCCA TCCCGCATCT GACCACCAGC GAAATGGATT 3720

TTTGCATCGA GCTGGGTAAT AAGCGTTGGC AATTTAACCG CCAGTCAGGC TTTCTTTCAC 3780

AGATGTGGAT TGGCGATAAA AAACAACTGC TGACGCCGCT GCGCGATCAG TTCACCCGTG 3840

CACCGCTGGA TAACGACATT GGCGTAAGTG AAGCGACCCG CATTGACCCT AACGCCTGGG 3900

TCGAACGCTG GAAGGCGGCG GGCCATTACC AGGCCGAAGC AGCGTTGTTG CAGTGCACGG 3960

CAGATACACT TGCTGATGCG GTGCTGATTA CGACCGCTCA CGCGTGGCAG CATCAGGGGA 4020

AAACCTTATT TATCAGCCGG AAAACCTACC GGATTGATGG TAGTGGTCAA ATGGCGATTA 4080

FIGURE 2E

CCGTTGATGT TGAAGTGGCG AGCGATACAC CGCATCCGGC GCGGATTGGC CTGAACTGCC 4140

AGCTGGCGCA GGTAGCAGAG CGGGTAAACT GGCTCGGATT AGGGCCGCAA GAAAACTATC 4200

CCGACCGCCT TACTGCCGCC TGTTTTGACC GCTGGGATCT GCCATTGTCA GACATGTATA 4260

CCCCGTACGT CTTCCCGAGC GAAAACGGTC TGCGCTGCGG GACGCGCGAA TTGAATTATG 4320

GCCCACACCA GTGGCGCGGC GACTTCCAGT TCAACATCAG CCGCTACAGT CAACAGCAAC 4380

TGATGGAAAC CAGCCATCGC CATCTGCTGC ACGCGGAAGA AGGCACATGG CTGAATATCG 4440

ACGGTTTCCA TATGGGGATT GGTGGCGACG ACTCCTGGAG CCCGTCAGTA TCGGCGGAAT 4500

TACAGCTGAG CGCCGGTCGC TACCATTACC AGTTGGTCTG GTGTCAAAAA TAATAATAAC 4560

CGGGCAGGCC ATGTCTGCCC GTATTTCGCG TAAGGAAATC CATTATGTAC TATTTAAAAA 4620

ACACAAACTT TTGGATGTTC GGTTTATTCT TTTTCTTTTA CTTTTTTATC ATGGGAGCCT 4680

ACTTCCCGTT TTTCCCGATT TGGCTACATG ACATCAACCA TATCAGCAAA AGTGATACGG 4740

GTATTATTTT TGCCGCTATT TCTCTGTTCT CGCTATTATT CCAACCGCTG TTTGGTCTGC 4800

TTTCTGACAA ACTCGGCCTC GACTCTAGGC GGCCGCGGGG ATCCAGACAT GATAAGATAC 4860

ATTGATGAGT TTGGACAAAC CACAACTAGA ATGCAGTGAA AAAAATGCTT TATTTGTGAA 4920

ATTTGTGATG CTATTGCTTT ATTTGTAACC ATTATAAGCT GCAATAAACA AGTTAACAAC 4980

AACAATTGCA TTCATTTTAT GTTTCAGGTT CAGGGGGAGG TGTGGGAGGT TTTTTCGGAT 5040

CCTCTAGAGT CGAGTAGATA AGTAGCATGG CGGGTTAATC ATTAACTACA AGGAACCCCT 5100

FIGURE 2F

AGTGATGGAG TTGGCCACTC CCTCTCTGCG CGCTCGCTCG CTCACTGAGG CCGGGCGACC 5160

AAAGGTCGCC CGACGCCCGG GCTTTGCCCG GGCGGCCTCA GTGAGCGAGC GAGCGCGCAG 5220

CAGATCTGGA AGGTGCTGAG GTACGATGAG ACCCGCACCA GGTGCAGACC CTGCGAGTGT 5280

GGCGGTAAAC ATATTAGGAA CCAGCCTGTG ATGCTGGATG TGACCGAGGA GCTGAGGCCC 5340

GATCACTTGG TGCTGGCCTG CACCCGCGCT GAGTTTGGCT CTAGCGATGA AGATACAGAT 5400

TGAGGTACTG AAATGTGTGG GCGTGGCTTA AGGGTGGGAA AGAATATATA AGGTGGGGGT 5460

CTTATGTAGT TTTGTATCTG TTTTGCAGCA GCCGCCGCCG CCATGAGCAC CAACTCGTTT 5520

GATGGAAGCA TTGTGAGCTC ATATTTGACA ACGCGCATGC CCCCATGGGC CGGGGTGCGT 5580

CAGAATGTGA TGGGCTCCAG CATTGATGGT CGCCCCGTCC TGCCCGCAAA CTCTACTACC 5640

TTGACCTACG AGACCGTGTC TGGAACGCCG TTGGAGACTG CAGCCTCCGC CGCCGCTTCA 5700

GCCGCTGCAG CCACCGCCCG CGGGATTGTG ACTGACTTTG CTTTCCTGAG CCCGCTTGCA 5760

AGCAGTGCAG CTTCCCGTTC ATCCGCCCGC GATGACAAGT TGACGGCTCT TTTGGCACAA 5820

TTGGATTCTT TGACCCGGGA ACTTAATGTC GTTTCTCAGC AGCTGTTGGA TCTGCGCCAG 5880

CAGGTTTCTG CCCTGAAGGC TTCCTCCCCT CCCAATGCGG TTTAAAACAT AAATAAAAAA 5940

CCAGACTCTG TTTGGATTTG GATCAAGCAA GTGTCTTGCT GTCTTTATTT AGGGGTTTTG 6000

CGCGCGCGGT AGGCCCGGGA CCAGCGGTCT CGGTCGTTGA GGGTCCTGTG TATTTTTTCC 6060

AGGACGTGGT AAAGGTGACT CTGGATGTTC AGATACATGG GCATAAGCCC GTCTCTGGGG 6120

FIGURE 2G

TGGAGGTAGC ACCACTGCAG AGCTTCATGC TGCGGGGTGG TGTTGTAGAT GATCCAGTCG 6180

TAGCAGGAGC GCTGGGCGTG GTGCCTAAAA ATGTCTTTCA GTAGCAAGCT GATTGCCAGG 6240

GGCAGGCCCT TGGTGTAAGT GTTTACAAAG CGGTTAAGCT GGGATGGGTG CATACGTGGG 6300

GATATGAGAT GCATCTTGGA CTGTATTTTT AGGTTGGCTA TGTTCCCAGC CATATCCCTC 6360

CGGGGATTCA TGTTGTGCAG AACCACCAGC ACAGTGTATC CGGTGCACTT GGGAAATTTG 6420

TCATGTAGCT TAGAAGGAAA TGCGTGGAAG AACTTGGAGA CGCCCTTGTG ACCTCCAAGA 6480

TTTTCCATGC ATTCGTCCAT AATGATGGCA ATGGGCCCAC GGGCGGCGGC CTGGGCGAAG 6540

ATATTTCTGG GATCACTAAC GTCATAGTTG TGTTCCAGGA TGAGATCGTC ATAGGCCATT 6600

TTTACAAAGC GCGGGCGGAG GGTGCCAGAC TGCGGTATAA TGGTTCCATC CGGCCCAGGG 6660

GCGTAGTTAC CCTCACAGAT TTGCATTTCC CACGCTTTGA GTTCAGATGG GGGGATCATG 6720

TCTACCTGCG GGGCGATGAA GAAAACGGTT TCCGGGGTAG GGGAGATCAG CTGGGAAGAA 6780

AGCAGGTTCC TGAGCAGCTG CGACTTACCG CAGCCGGTGG GCCCGTAAAT CACACCTATT 6840

ACCGGGTGCA ACTGGTAGTT AAGAGAGCTG CAGCTGCCGT CATCCCTGAG CAGGGGGGCC 6900

ACTTCGTTAA GCATGTCCCT GACTCGCATG TTTTCCCTGA CCAAATCCGC CAGAAGGCGC 6960

TCGCCGCCCA GCGATAGCAG TTCTTGCAAG GAAGCAAAGT TTTTCAACGG TTTGAGACCG 7020

TCCGCCGTAG GCATGCTTTT GAGCGTTTGA CCAAGCAGTT CCAGGCGGTC CCACAGCTCG 7080

GTCACCTGCT CTACGGCATC TCGATCCAGC ATATCTCCTC GTTTCGCGGG TTGGGGCGGC 7140

FIGURE 2H

TTTCGCTGTA CGGCAGTAGT CGGTGCTCGT CCAGACGGGC CAGGGTCATG TCTTTCCACG 7200

GGCGCAGGGT CCTCGTCAGC GTAGTCTGGG TCACGGTGAA GGGGTGCGCT CCGGGCTGCG 7260

CGCTGGCCAG GGTGCGCTTG AGGCTGGTCC TGCTGGTGCT GAAGCGCTGC CGGTCTTCGC 7320

CCTGCGCGTC GGCCAGGTAG CATTTGACCA TGGTGTCATA GTCCAGCCCC TCCGCGGCGT 7380

GGCCCTTGGC GCGCAGCTTG CCCTTGGAGG AGGCGCCGCA CGAGGGGCAG TGCAGACTTT 7440

TGAGGGCGTA GAGCTTGGGC GCGAGAAATA CCGATTCCGG GGAGTAGGCA TCCGCGCCGC 7500

AGGCCCCGCA GACGGTCTCG CATTCCACGA GCCAGGTGAG CTCTGGCCGT TCGGGGTCAA 7560

AAACCAGGTT TCCCCCATGC TTTTTGATGC GTTTCTTACC TCTGGTTTCC ATGAGCCGGT 7620

GTCCACGCTC GGTGACGAAA AGGCTGTCCG TGTCCCCGTA TACAGACTTG AGAGGCCTGT 7680

CCTCGACCGA TGCCCTTGAG AGCCTTCAAC CCAGTCAGCT CCTTCCGGTG GGCGCGGGGC 7740

ATGACTATCG TCGCCGCACT TATGACTGTC TTCTTTATCA TGCAACTCGT AGGACAGGTG 7800

CCGGCAGCGC TCTGGGTCAT TTTCGGCGAG GACCGCTTTC GCTGGAGCGC GACGATGATC 7860

GGCCTGTCGC TTGCGGTATT CGGAATCTTG CACGCCCTCG CTCAAGCCTT CGTCACTGGT 7920

CCCGCCACCA AACGTTTCGG CGAGAAGCAG GCCATTATCG CCGGCATGGC GGCCGACGCG 7980

CTGGGCTACG TCTTGCTGGC GTTCGCGACG CGAGGCTGGA TGGCCTTCCC CATTATGATT 8040

CTTCTCGCTT CCGGCGGCAT CGGGATGCCC GCGTTGCAGG CCATGCTGTC CAGGCAGGTA 8100

GATGACGACC ATCAGGGACA GCTTCAAGGA TCGCTCGCGG CTCTTACCAG CCTAACTTCG 8160

FIGURE 2I

ATCACTGGAC CGCTGATCGT CACGGCGATT TATGCCGCCT CGGCGAGCAC ATGGAACGGG 8220

TTGGCATGGA TTGTAGGCGC CGCCCTATAC CTTGTCTGCC TCCCCGCGTT GCGTCGCGGT 8280

GCATGGAGCC GGGCCACCTC GACCTGAATG GAAGCCGGCG GCACCTCGCT AACGGATTCA 8340

CCACTCCAAG AATTGGAGCC AATCAATTCT TGCGGAGAAC TGTGAATGCG CAAACCAACC 8400

CTTGGCAGAA CATATCCATC GCGTCCGCCA TCTCCAGCAG CCGCACGCGG CGCATCTCGG 8460

GCAGCGTTGG GTCCTGGCCA CGGGTGCGCA TGATCGTGCT CCTGTCGTTG AGGACCCGGC 8520

TAGGCTGGCG GGGTTGCCTT ACTGGTTAGC AGAATGAATC ACCGATACGC GAGCGAACGT 8580

GAAGCGACTG CTGCTGCAAA ACGTCTGCGA CCTGAGCAAC AACATGAATG GTCTTCGGTT 8640

TCCGTGTTTC GTAAAGTCTG GAAACGCGGA AGTCAGCGCC CTGCACCATT ATGTTCCGGA 8700

TCTGCATCGC AGGATGCTGC TGGCTACCCT GTGGAACACC TACATCTGTA TTAACGAAGC 8760

CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG 8820

GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT 8880

CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC TGGTAACAGG 8940

ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC 9000

GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA 9060

AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG TGGTTTTTTT 9120

GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC TTTGATCTTT 9180

FIGURE 2J

TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT GGTCATGAGA 9240

TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC 9300

TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT 9360

ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA 9420

ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA 9480

CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC CGAGCGCAGA 9540

AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA 9600

GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTGC AGGCATCGTG 9660

GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA 9720

GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT 9780

GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT GCATAATTCT 9840

CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA 9900

TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT 9960

ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA 10020

AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC 10080

AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG 10140

CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC 10200

FIGURE 2K

CTTTTTCAAT ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT
10260

GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA
10320

CCTGACGTCT AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG
10380

AGGCCCTTTC GTCTTCAA
10398

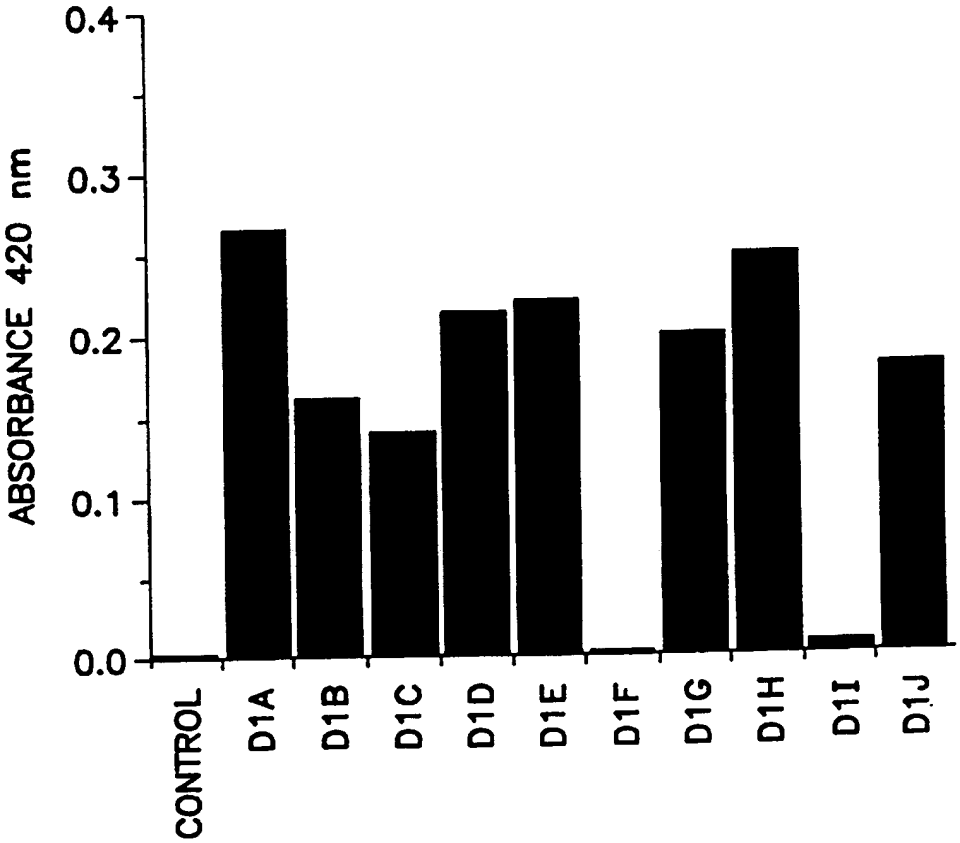

ABSORBANCE 420 nm
0.4
0.3
0.2
0.1
0.0
CONTROL
D1A
D1B
D1C
D1D
D1E
D1F
D1G
D1H
D1I
D1J

FIG. 3

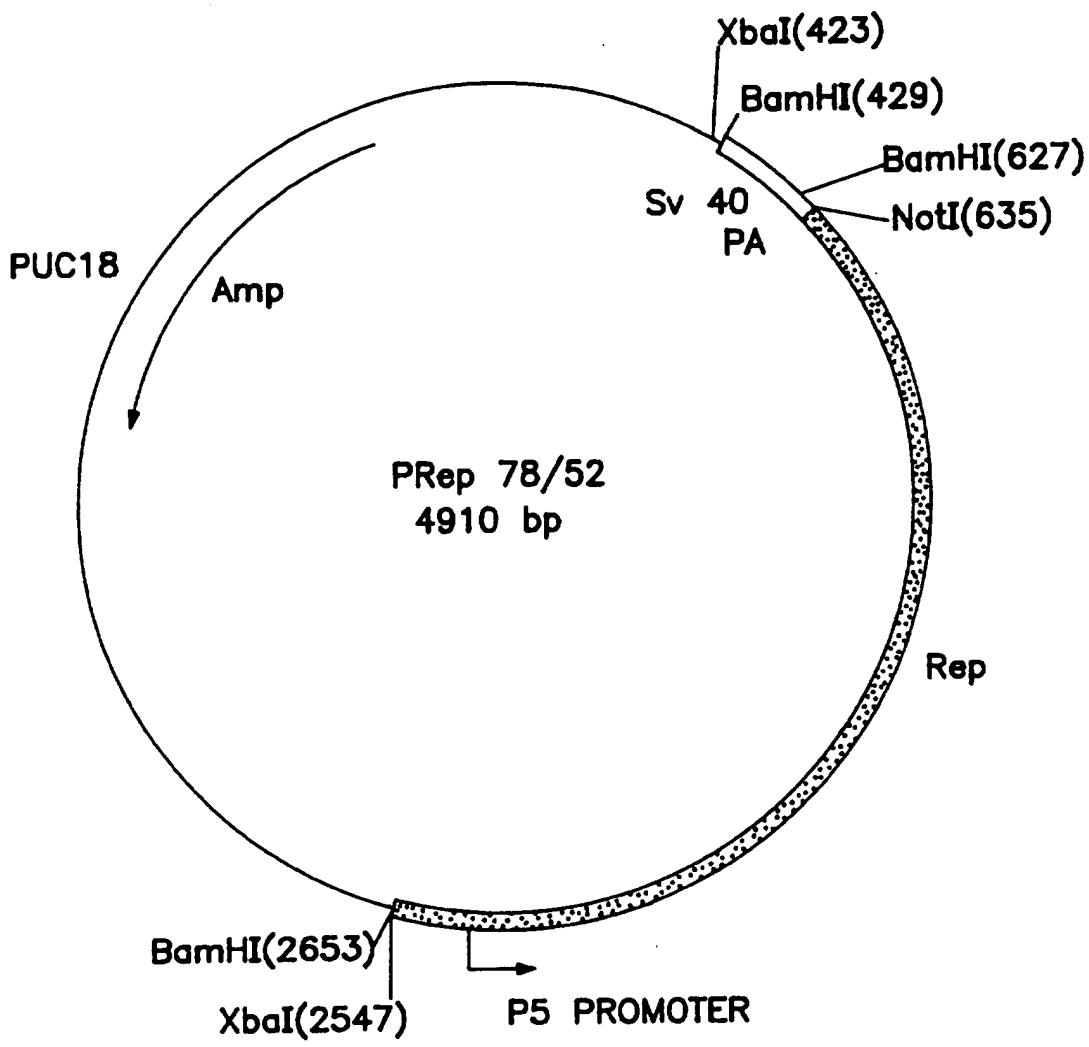

XbaI(423)
BamHI(429)
BamHI(627)
NotI(635)
Sv 40
PA
PUC18
Amp
PRep 78/52
4910 bp
Rep
BamHI(2653)
XbaI(2547)
P5 PROMOTER

FIG. 4

FIGURE 5A

TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA 60

CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG 120

TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC 180

ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC 240

ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT 300

TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT 360

TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAA GCTTGCATGC CTGCAGGTCG 420

ACTCTAGAGG ATCCGAAAAA ACCTCCCACA CCTCCCCCTG AACCTGAAAC ATAAAATGAA 480

TGCAATTGTT GTTGTTAACT TGTTTATTGC AGCTTATAAT GGTTACAAAT AAAGCAATAG 540

CATCACAAAT TTCACAAATA AAGCATTTTT TTCACTGCAT TCTAGTTGTG GTTTGTCCAA 600

ACTCATCAAT GTATCTTATC ATGTCTGGAT CCCCGCGGCC GCCAAATCAT TTATTGTTCA 660

AAGATGCAGT CATCCAAATC CACATTGACC AGATCGCAGG CAGTGCAAGC GTCTGGCACC 720

TTTCCCATGA TATGATGAAT GTAGCACAGT TTCTGATACG CCTTTTTGAC GACAGAAACG 780

GGTTGAGATT CTGACACGGG AAAGCACTCT AAACAGTCTT TCTGTCCGTG AGTGAAGCAG 840

ATATTTGAAT TCTGATTCAT TCTCTCGCAT TGTCTGCAGG GAAACAGCAT CAGATTCATG 900

CCCACGTGAC GAGAACATTT GTTTTGGTAC CTGTCTGCGT AGTTGATCGA AGCTTCCGCG 960

TCTGACGTCG ATGGCTGCGC AACTGACTCG CGCACCCGTT TGGGCTCACT TATATCTGCG 1020

FIGURE 5B

TCACTGGGGG CGGGTCTTTT CTTGGCTCCA CCCTTTTTGA CGTAGAATTC ATGCTCCACC 1080

TCAACCACGT GATCCTTTGC CCACCGGAAA AAGTCTTTGA CTTCCTGCTT GGTGACCTTC 1140

CCAAAGTCAT GATCCAGACG GCGGGTGAGT TCAAATTTGA ACATCCGGTC TTGCAACGGC 1200

TGCTGGTGTT CGAAGGTCGT TGAGTTCCCG TCAATCACGG CGCACATGTT GGTGTTGGAG 1260

GTGACGATCA CGGGAGTCGG GTCTATCTGG GCCGAGGACT TGCATTTCTG GTCCACGCGC 1320

ACCTTGCTTC CTCCGAGAAT GGCTTTGGCC GACTCCACGA CCTTGGCGGT CATCTTCCCC 1380

TCCTCCCACC AGATCACCAT CTTGTCGACA CAGTCGTTGA AGGGAAAGTT CTCATTGGTC 1440

CAGTTTACGC ACCCGTAGAA GGGCACAGTG TGGGCTATGG CCTCCGCGAT GTTGGTCTTC 1500

CCGGTAGTTG CAGGCCCAAA CAGCCAGATG GTGTTCCTCT TGCCGAACTT TTTCGTGGCC 1560

CATCCCAGAA AGACGGAAGC CGCATATTGG GGATCGTACC CGTTTAGTTC CAAAATTTTA 1620

TAAATCCGAT TGCTGGAAAT GTCCTCCACG GGCTGCTGGC CCACCAGGTA GTCGGGGGCG 1680

GTTTTAGTCA GGCTCATAAT CTTTCCCGCA TTGTCCAAGG CAGCCTTGAT TTGGGACCGC 1740

GAGTTGGAGG CCGCATTGAA GGAGATGTAT GAGGCCTGGT CCTCCTGGAT CCACTGCTTC 1800

TCCGAGGTAA TCCCCTTGTC CACGAGCCAC CCGACCAGCT CCATGTACCT GGCTGAAGTT 1860

TTTGATCTGA TCACCGGCGC ATCAGAATTG GGATTCTGAT TCTCTTTGTT CTGCTCCTGC 1920

GTCTGCGACA CGTGCGTCAG ATGCTGCGCC ACCAACCGTT TACGCTCCGT GAGATTCAAA 1980

CAGGCGCTTA AATACTGTTC CATATTAGTC CACGCCCACT GGAGCTCAGG CTGGGTTTTG 2040

FIGURE 5C

GGGAGCAAGT AATTGGGGAT GTAGCACTCA TCCACCACCT TGTTCCCGCC TCCGGCGCCA
2100

TTTCTGGTCT TTGTGACCGC GAACCAGTTT GGCAAAGTCG GCTCGATCCC GCGGTAAATT
2160

CTCTGAATCA GTTTTTCGCG AATCTGACTC AGGAAACGTC CCAAAACCAT GGATTTCACC
2220

CCGGTGGTTT CCACGAGCAC GTGCATGTGG AAGTAGCTCT CTCCCTTCTC AAATTGCACA
2280

AAGAAAAGGG CCTCCGGGGC CTTACTCACA CGGCGCCATT CCGTCAGAAA GTCGCGCTGC
2340

AGCTTCTCGG CCACGGTCAG GGGTGCCTGC TCAATCAGAT TCAGATCCAT GTCAGAATCT
2400

GGCGGCAACT CCCATTCCTT CTCGGCCACC CAGTTCACAA AGCTGTCAGA AATGCCGGGC
2460

AGATGCCCGT CAAGGTCGCT GGGGACCTTA ATCACAATCT CGTAAAACCC CGGCATGGCG
2520

GCTGCGCGTT CAAACCTCCC GCTTCAAAAT GGAGACCCTG CGTGCTCACT CGGGCTTAAA
2580

TACCCAGCGT GACCACATGG TGTCGCAAAA TGTCGCAAAA CACTCACGTG ACCTCTAATA
2640

CAGGACTCTA GAGGATCCCC GGGTACCGAG CTCGAATTCG TAATCATGGT CATAGCTGTT
2700

TCCTGTGTGA AATTGTTATC CGCTCACAAT TCCACACAAC ATACGAGCCG GAAGCATAAA
2760

GTGTAAAGCC TGGGGTGCCT AATGAGTGAG CTAACTCACA TTAATTGCGT TGCGCTCACT
2820

GCCCGCTTTC CAGTCGGGAA ACCTGTCGTG CCAGCTGCAT TAATGAATCG GCCAACGCGC
2880

GGGGAGAGGC GGTTTGCGTA TTGGGCGCTC TTCCGCTTCC TCGCTCACTG ACTCGCTGCG
2940

CTCGGTCGTT CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC
3000

CACAGAATCA GGGGATAACG CAGGAAAGAA CATGTGAGCA AAAGGCCAGC AAAAGGCCAG
3060

FIGURE 5D

GAACCGTAAA AAGGCCGCGT TGCTGGCGTT TTTCCATAGG CTCCGCCCCC CTGACGAGCA 3120

TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG ACAGGACTAT AAAGATACCA 3180

GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG 3240

ATACCTGTCC GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT TCTCATAGCT CACGCTGTAG 3300

GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT 3360

TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA 3420

CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG 3480

CGGTGCTACA GAGTTCTTGA AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT 3540

TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA GCTCTTGATC 3600

CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG 3660

CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG 3720

GAACGAAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA TCAAAAAGGA TCTTCACCTA 3780

GATCCTTTTA AATTAAAAAT GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG 3840

GTCTGACAGT TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG 3900

TTCATCCATA GTTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC 3960

ATCTGGCCCC AGTGCTGCAA TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC 4020

AGCAATAAAC CAGCCAGCCG GAAGGGCCGA GCGCAGAAGT GGTCCTGCAA CTTTATCCGC 4080

FIGURE 5E

CTCCATCCAG TCTATTAATT GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG
4140

TTTGCGCAAC GTTGTTGCCA TTGCTACAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT
4200

GGCTTCATTC AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG
4260

CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT
4320

GTTATCACTC ATGGTTATGG CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG
4380

ATGCTTTTCT GTGACTGGTG AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG
4440

ACCGAGTTGC TCTTGCCCGG CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT
4500

AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT
4560

GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC
4620

TTTCACCAGC GTTTCTGGGT GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT
4680

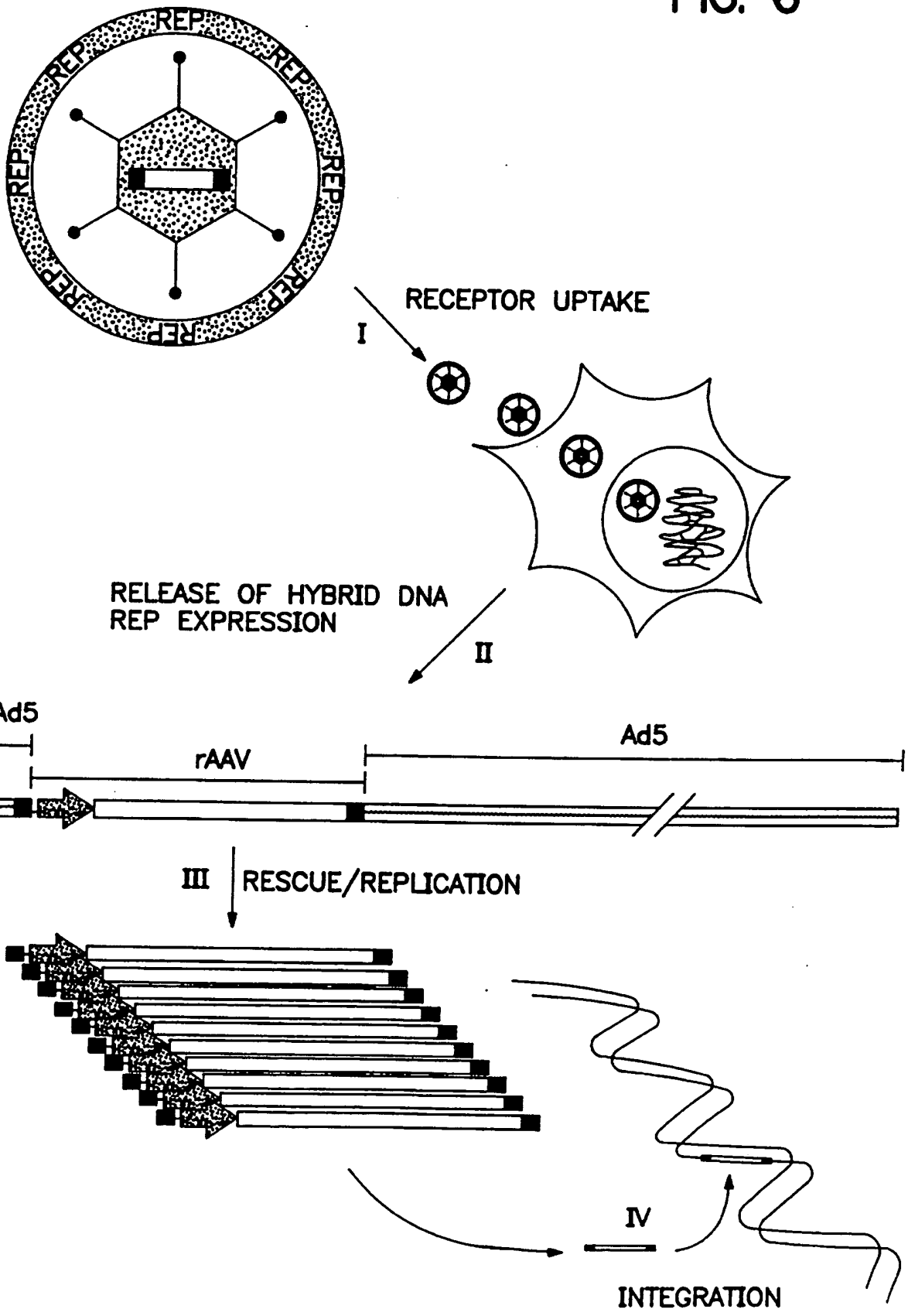
AAGGGCGACA CGGAAATGTT GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT
4740

TTATCAGGGT TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA
4800

AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG AAACCATTAT
4860

TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC
4910

FIG. 6


REP
REP
REP
REP
REP
REP
REP
REP
RECEPTOR UPTAKE
I
RELEASE OF HYBRID DNA
REP EXPRESSION
II
Ad5
rAAV
Ad5
III
RESCUE/REPLICATION
IV
INTEGRATION

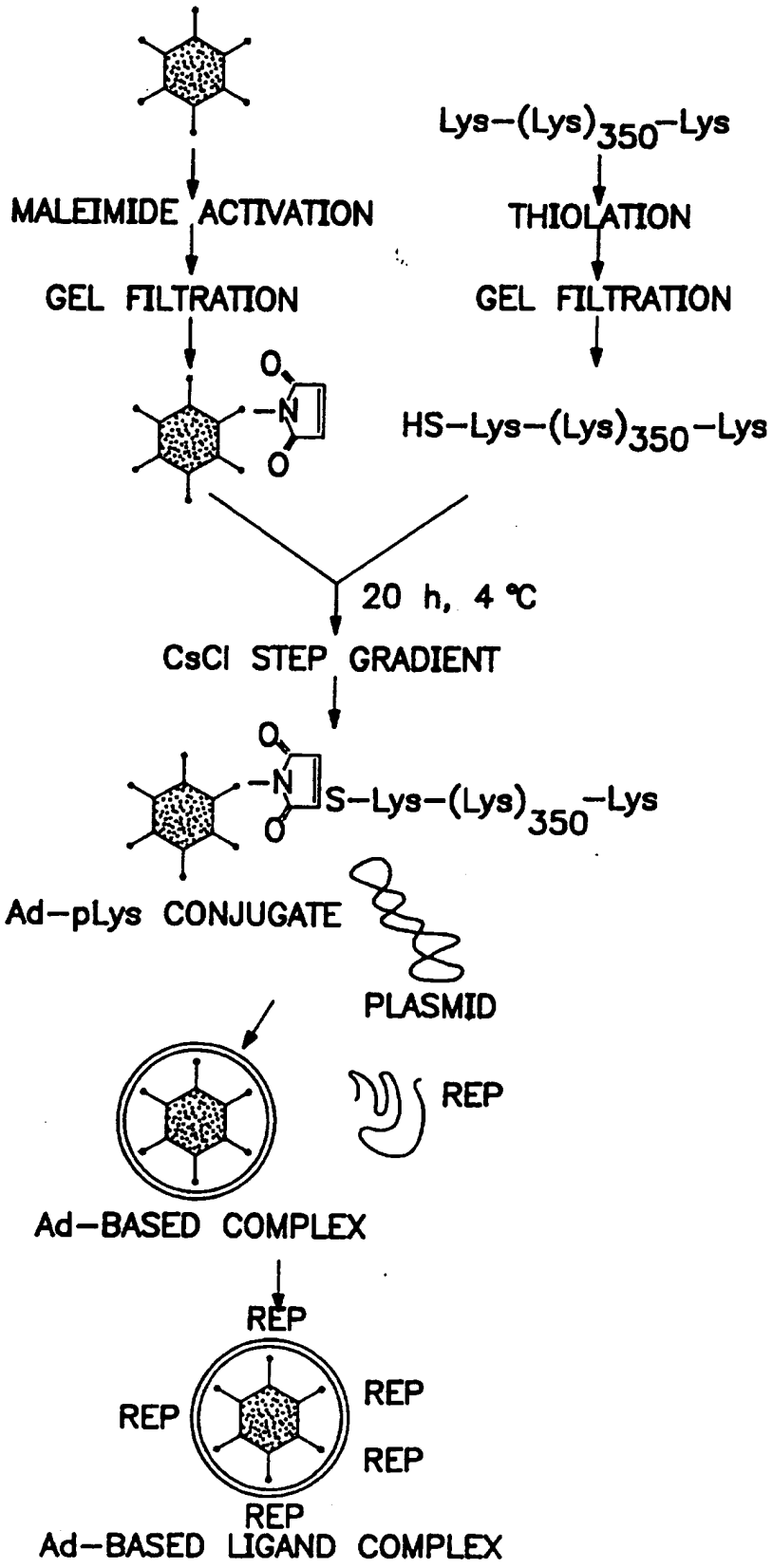

Lys–(Lys)350–Lys
MALEIMIDE ACTIVATION
THIOLATION
GEL FILTRATION
GEL FILTRATION
HS–Lys–(Lys)350–Lys
20 h, 4 ℃
CsCl STEP GRADIENT
S–Lys–(Lys)350–Lys
Ad–pLys CONJUGATE
PLASMID
REP
Ad–BASED COMPLEX
REP
REP
REP
REP
REP
Ad–BASED LIGAND COMPLEX

FIG. 7